Endogenous Cannabinoid Regulation of the Hypothalamic-Pituitary-Adrenal Axis and of Neuroendocrine Reactivity to Acute and Repeated Stress

by

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Abstract

Deleterious effects of stress contribute to many mind/body ills and precipitate substantial individual and societal burden. The endogenous cannabinoid (endocannabinoid, eCB) system is widely expressed in the brain and body, and contributes to psychoneuroendocrine regulation. Inhibitory CB1 receptors on neurons afford the “toning down” of conscious experience that makes cannabis a popular relaxant. In normal waking consciousness, the eCB ligands anandamide and 2-AG both bind at this predominantly presynaptic receptor and function as a negative feedback mechanism for neurotransmission. Peripheral eCB activity is less explored in stress research and is of interest in metabolic regulation. Excessive elevations of stress-induced cortisol and excitatory neurotransmission are interacting pathological influences that may be regulated by the eCB system in both acute and repeated stress. We initially demonstrated that systemic antagonism of CB1 receptors potentiates some measures of neural and hypothalamic-pituitary-adrenal (HPA) axis response to acute loud noise stress, and that antagonism of CB1 receptors alone directly stimulated activity in a select subset of neural regions, and elevated plasma corticosterone (CORT, the rodent equivalent of cortisol). We have explored involvement of CB1 receptors in inhibition of central and peripheral psychoneuroendocrine stress reactivity in acute and repeated stress, and in contributing to constitutive tonic inhibition in regions including the amygdala and adrenal glands.

I dedicate this extended effort to the rain, and through the rain to everything.

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Cosmic Casey O'Neill, PhD.

(We will have quite the battle over whether or not she'll take on my last name next year.)

Casey, my heart. You redefine reality and fascinate me. I'm going to be bold and tell the truth. When we were dating long distance for the first year, you and I would "talk" online every night until it was time to go to sleep. We would keep talking in dream, and sometimes when we checked in with each other first thing the next morning, you could tell me what we had just said to each other from my perspective in the dream. Together we conquered space and proved it beyond material. I worked quite hard to prove my perceptual capacity with this special perception of ours in mind. Will I come across as too weird if I state in this permanent record that you taught me yoga so deeply that we could take on each other's physical and psychological frustrations remotely? There's no sweeter display of young love than to try to take on your partner's hangover so they don't have to feel as bad the morning after partying like a student. I feel like we owe it to PubMed to pass on our knowledge that if you find your soul mate, it doesn't matter which one holds the mudra, because both of you benefit from the energetic actions.

Knowing my deep Love for Science, and my deep Love for Spirit, please accept this bold display of my love for you as worth any raised eyebrows if someone happens upon this dedication one day. I very intentionally bare my soul in my dissertation by writing this. This dissertation is my masterpiece, and my offering to the Universe. You are the only other one who knows the totality of my effort and heartache that have gone into this body of work, and having just fought 7 years to prove my scientific legitimacy, I take a bit of a risk by writing this. Namaste.

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"Strange ode to nature
He built it in the woods
Brought it to his doctor
Just to see if it was any good
The doctor just misunderstood."

-Trevor Garrod, the music man (TLG, NFA)

"Slim just dreamed over his head."

-Jack Kerouac, The Dharma Bums.

"45 And I'll stride freely through wide open spaces as I look for your truth and your wisdom; 46 Then I'll tell the world what I find, speak out boldly in public, unembarrassed."

-Psalm 119: 45-46

"I am convinced that the progress or decline of humanity rests very largely with educators and teachers, who therefore have a tremendous responsibility"

-His Holiness the 14th Dalai Lama

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Chapter 1

General Introduction

This dissertation work was designed based on an understanding that chronic stress is broadly damaging to health (McEwen, 1998). Repeated stress is almost unavoidable in modern life (McEwen, 1998), and can lead to habituation and sensitization of psychoneuroendocrine stress reactivity (Herman, 2013). One's protective ability to habituate to repeatedly experienced stressors is dependent on factors including stressor intensity and stress-reactivity (Herman, 2013). Acute psychoneuroendocrine stress-reactivity is of interest considering the involvement of this reactivity in stressors that become repeatedly experienced stressors (Sapolsky, 1996). Many central and peripheral systems and structures in the body contribute to acute psychoneuroendocrine stress-reactivity (McEwen, 1998), and some of these components may have both tonic and phasic responsibilities. The second chapter of this dissertation includes system-wide examination of the potential role of the endogenous cannabinoid system in acute stress reactivity. The third chapter includes examination of peripheral and tonic involvement of the eCB system in psychoneuroendocrine regulation. The fourth chapter includes examination of a potential role of the eCB system in mediating habituation and sensitization of psychoneuroendocrine reactivity to repeated stressors.

Introduction

Stress contributes to the initiation and maintenance of a variety of disrupted states of physical and psychological health such as anxiety, depression, post-traumatic stress disorder (PTSD), addiction, psychosis, heart disease, diabetes, asthma, and autoimmune disorders (Barnes, 2013; Herman, Ostrander, Mueller, & Figueiredo, 2005; Koob, 2009; McEwen, 1998; 2006; Mittal et al., 2013; Rodwin, Spruill, & Ladapo, 2013). A substantial amount of modern human life stressors are psychologically or emotionally challenging and are disruptive due to their chronic nature (McEwen, 2006). The protective response of an organism to acute psychological stressors includes reactions in multiple cognitive and physiological systems collectively functioning to increase awareness and available energy to respond to the challenge (de Kloet, Joëls, & Holsboer, 2005; Sapolsky, 2000). Elements of this protective response to acute stress include increased central and peripheral levels of glucocorticoids and catecholamines and increased psychological arousal (McEwen, 2006). Repeated or prolonged psychological stress can result in increased emotional reactivity as well as disruption of physical systems due to the repeated activation of neural and endocrine systems responsive to stress. Often, stressors that are repeatedly experienced result in gradual reduction of neural and physical reactions in an adaptive process termed habituation (Campeau, Liberzon, Morilak, & Ressler, 2011; Grissom & Bhatnagar, 2009). Habituation of responsiveness to familiar stressors is an important adaptation that can limit the disruptive influence of stress (Herman, 2013). Some types of stress result in gradually increasing reaction to familiar or unfamiliar stressors in a process referred to as sensitization (García-Iglesias et al., 2013). Though sensitization of physical and

psychoemotional reactivity can be adaptive in short-term situations benefited by facilitated responses, it may also result in increased accumulation of stress-related damage and contribute to disrupted health (McEwen, 2004).

It has been observed in stress research that the intensity of a repeatedly experienced stressor can determine whether response patterns favor habituation or sensitization (Pitman, Ottenweller, & Natelson, 1990). In a normal state of health, the intensity of a stressor typically determines the magnitude of the neuroendocrine responses elicited, which are in proportion to the severity of the threat (Charmandari, Tsigos, & Chrousos, 2005). The damaging effects of excessive glucocorticoid exposure and excitatory neurotransmission are known to be pathological influences that can lead to a sensitization of psychoneuroendocrine reactivity, which can contribute to the development of a wide variety of psychological and physical disruptions (de Kloet et al., 2005; McEwen, 2004; McEwen & Stellar, 1993; Sapolsky, 1996). The mechanisms of habituation to stress are not well understood, but it seems likely that they are sensitive to disruption by the high levels of glucocorticoids and excitatory neurotransmission that result from repeated higher intensity stress and that are known to lead to sensitization (McEwen, 2004). The physical mechanisms that contribute to inhibitory regulation of glucocorticoid and excitatory neurotransmitter activity are likely an important regulatory system that is damaged by stress, allowing an initiation point to sensitization and disrupted habituation. Strategies to improve, protect, or correct the functioning of various components of this hypothetical regulatory mechanism would be important for preventing or correcting stress-related dysfunction (McEwen & Stellar, 1993). The

endogenous cannabinoid system is a modulatory system that is expressed in central and peripheral tissues (Hill, Patel, et al., 2010b), which may allow for inhibitory influence of stress-induced excitatory neural activity and glucocorticoid release. The various inhibitory contributions of the eCB system on psychoneuroendocrine activity are the subject of the original research included in this dissertation.

Habituation and Sensitization

Habituation to repeatedly experienced stressors includes reduction of a variety of behavioral, neural, endocrine, and autonomic responses (Campeau, Dolan, Akil, & Watson, 2002; Campeau, Nyhuis, Sasse, Day, & Masini, 2008; Girotti et al., 2006; Grissom & Bhatnagar, 2009; Pfister & King, 1976) and most readily occurs in response to repeated homotypic stressor experiences. This reduction of responses is specific to the familiar stressor and has been reported to associate with normal or facilitated responding to novel heterotypic stressors (Armario, Gavaldà, & Martí, 1988; Bhatnagar & Dallman, 1998). The stressor-specific nature of habituation gives evidence that the responsible plasticity does not occur as general desensitization in structures regulating responses to stress, but the exact neural circuitry responsible for this plasticity is currently unknown. Disrupted habituation to stress has been reported in stress-related disorders of emotionality including: anxiety, depression, and PTSD (Brierley & Jamieson, 1974; Chattopadhyay, Cooke, Toone, & Lader, 1980; Lader et al., 1964; Thomson & Craighead, 2008). Along with emotional disorders, failure to habituate hypothalamic-pituitary-adrenal axis (HPA) responses to repeatedly experienced stress

could understandably contribute to many diseases relating to hypercortisolism, HPA axis dysregulation, inflammation, neurodegeneration, and disrupted circadian rhythm (Esch, Stefano, Fricchione, & Benson, 2002; Maury, Ramsey, & Bass, 2010; Sorrells & Sapolsky, 2007a). The involvement of chronic stress and disrupted ability to habituate to stress in a wide variety of conditions of impaired emotional and physical health support the necessity of further study of habituation. Improved understanding of the neural circuitry and processes responsible for habituation to stress may provide strategies to correct and enhance this ability in clinical populations, which would provide substantial improvement of treatment strategies.

In contrast to habituation of responses to repeated homotypic stress, some types of repeated stress result in sensitization of responses. Sensitization of stress reactivity can include increases in behavioral (Grissom, Kerr, & Bhatnagar, 2008), emotional (Fani et al., 2012; Stein, Simmons, Feinstein, & Paulus, 2007), neural (Aoife O'Donovan, Slavich, Epel, & Neylan, 2013), endocrine (Fernandes et al., 2002; J. D. Johnson et al., 2002), and autonomic responses (Konarska, Stewart, & McCarty, 1990) elicited by stress. Hypersensitivity to stress is thought to contribute to emotional disorders such as PTSD, depression, panic disorder and anxiety (Chopra et al., 2009; Heim, Newport, Mletzko, Miller, & Nemeroff, 2008; Stam, Bruijnzeel, & Wiegant, 2000). In experimental settings, this pattern is most reported in response to chronic variable, unpredictable, or uncontrollable stress. An important factor in sensitization to variable and unpredictable stressors may be a lack of habituation that leads to more cumulative influence of emotional and physical reactions to stress. Sensitization has been observed

in response to repeated homotypic stressors such as forced swim stress (Konarska et al., 1990), and a combination of immobilization, light and noise stress (Vogel & Jensh, 1988). The few reports of homotypic stressor sensitization provide evidence that the intensity of stressful experience is an important factor influencing the response to repeated exposure. In Konarska et al. (1990), rats exposed to 27 days of 30-minute sessions of forced swim expressed a sensitized response of stress-induced plasma norepinephrine (NE) if the stressor exposures included colder water (18^0 or 24^0 C), but habituation of this response if the water was warmer (34^0 C) during repeated exposures. The combination of immobilization, light, and noise stressors simultaneously used in Vogule and Jensh, (1988) resulted in a pattern of sensitized stress-induced corticosterone (CORT) secretion during the third week of daily exposures. All three of the stressors used in that study have been reported to result in habituation when used singularly (Campeau et al., 2002; Hauger, Lorang, Irwin, & Aguilera, 1990), suggesting that it was the increased impact of the combination of stressors that resulted in sensitization rather than habituation. More commonly reported than homotypic stressor sensitization is a pattern of maintained level of responding or resistance of habituation to repeated stress (Grissom et al., 2008; Pitman, Ottenweller, & Natelson, 1988; Sgoifo et al., 2002; Umemoto, Kawai, Ueyama, & Senba, 1997; Umemoto, Noguchi, Kawai, & Senba, 1994). A distinction of higher intensity stressors resulting in resistance of habituation has been noted (Cox, Hubbard, Lawler, Sanders, & Mitchell, 1985; Konarska et al., 1990; Natelson et al., 1988; Pitman et al., 1988), but it has yet to be determined exactly how stressor intensity influences the direction of responding of an

organism when exposed to repeated stress. Importantly, the variation in response patterns suggests that repeated stress results in a spectrum of reactivity to subsequent stressful experience in a range including habituation, maintenance, and sensitization of responding, depending on the intensity of the stressful experience.

In a context of modern life stress, which often includes continuous experience of stressors both successively and at the same time, it is important to understand the ways in which stressful experiences influence the responding to additional, novel stressors. A phenomenon often reported in research literature is a pattern of habituation to one stressor that is repeatedly experienced resulting in a sensitized response to novel, heterotypic stressors (reviewed in:(Armario, 2006; Grissom & Bhatnagar, 2009). The theoretical basis of this well-replicated pattern of responding to multiple stressors is that repeated stress results in neural plasticity or a stress-related memory trace that is facilitative to responding to novel stressors (Bhatnagar & Dallman, 1998). Increasingly well-accepted in stress habituation literature is an idea that repeated exposures to a stressor result in co-occurrence of competing processes of habituation and sensitization, in which habituation to the repeated stimulus is eventually expressed (Groves & Thompson, 1970). Observations of increased sensitivity to novel stressors after habituation of responses to a repeatedly experienced stressor may be driving the growing sentiment that habituation and sensitization always co-occur in repeated stress (Fernandes et al., 2002; Grissom, Iyer, Vining, & Bhatnagar, 2007; Spiga et al., 2009). It seems a mistake to interpret this pattern to indicate that the plasticity responsible for habituation of responding to repeated homotypic stress is sufficiently responsible for

expressions of heterotypic stressor sensitization. Reports of distinct temporal patterns of sensitization and habituation (Masini et al., 2006; M. S. Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008a), novel stressor sensitization after repeated exposure to non-habituating stressors (Kanai et al., 2007; Marin, Cruz, & Planeta, 2007), and homotypic stressor sensitization (García-Iglesias et al., 2013; Konarska et al., 1990; Vogel & Jensh, 1988) support mechanisms of stressor sensitization that are independent of the plasticity responsible for habituation. Habituation of responding to a familiar stressor often does not result in heterotypic stressor sensitization (Armario, Lopez-Calderon, Jolin, & Balasch, 1986; Babb, Masini, Day, & Campeau, 2014; Melia, Ryabinin, Schroeder, Bloom, & Wilson, 1994), but rather maintenance of stress-reactivity compared to the acute response of conspecifics without recent stress history. Also, our lab has stumbled onto unexpected preliminary evidence that habituation to mild stress can result in a reduction in responding to subsequent heterotypic mild stress (unpublished). I feel that emphasis on the development of homotypic stressor habituation as a process directly related to or responsible for a state of novel stressor sensitivity is misguided. Patterns of reactivity to novel stressors after prior exposure to repeated stress can occur in a spectrum including: decreased, maintained, and facilitated.

It is possible that, as in response patterns to repeated homotypic stress, the intensity of stressful experience is a main factor influencing the state of novel stressor reactivity of an organism. Inconsistency in states of sensitivity to novel stress observed after different regimens of repeated habituating stress may be better explained by the

variation in cumulative negative effects resulting from different stressor paradigms. For example, the stress-related wear and tear resulting from 6 weeks of daily, 1hr sessions of forced swim (which has been reported to slowly result in partial habituation in (Cox et al., 1985)) should be much greater than that from 6 weeks of daily saline injection or handling (stressors which result in more rapid and complete habituation as in (Ryabinin, Wang, & Finn, 1999)).

The working definition of habituation in stress research could benefit from elaboration to include acknowledgement of the magnitude of habituation and resulting level of sustained responding, rather than the often-used criterion of significant reduction compared to initial level of responding. An important clarification in the relationship of stressor habituation and sensitization could be that partial habituation to a repeatedly experienced stressor may still allow for damaging influence from repeated emotional and physical arousal that support the development of a state of hypersensitivity to novel stress. An equally important extension of this idea is that experiences of mild, readily adaptable stressors would likely have a different effect on an organism. Much like environmental enrichment, the experience of mild stressors may provide important stimulation that is fortifying and protective (Belz, Kennell, Czambel, Rubin, & Rhodes, 2003). From this perspective, it will be important to examine more closely the relationship of repeated stress to the processes of habituation and sensitization of reactivity with a critical emphasis on the role of stressor intensity. Advancement in the understanding of the neural systems involved in habituating to repeated stress, and aspects of these systems that are sensitive to the damaging

influence of stress, would be importantly applicable to the clinical treatment of disorders involving self-perpetuating sensitivity to stress.

The Endogenous Cannabinoid System as a Regulator of Psychoneuroendocrine Activity in Acute and Repeated Stress

Several lines of recent evidence point to the involvement of the endogenous cannabinoid (eCB) system in negatively regulating psychological and physical reactivity to stress (Hill & McEwen, 2010). This modulatory system is widely expressed in the brain and body and participates in activity-dependent negative feedback fine tuning of activity in a variety of cell types including neurons and immune cells (microglia: (Benito et al., 2008; Fisar, 2009)). The two main endogenous ligands, N-arachidonylethanolamine (AEA or "anandamide" (Devane et al., 1992)) and 2-arachidonylglycerol (2AG) (Sugiura et al., 1995), are agonists at inhibitory g-protein coupled (Gi/o) CB1 and CB2 receptors (Herkenham et al., 1991; Stella, Schweitzer, & Piomelli, 1997). Of particular interest in stress reactivity are inhibitory CB1 receptors on presynaptic terminals of neurons, and the activity-dependent synthesis of AEA and 2AG in the post-synaptic neuron. Once synthesized, these lipid cannabinoid ligands diffuse across the cell membrane and retrogradely bind at presynaptic CB1 receptors, functioning to inhibit calcium influx and the further release of neurotransmitters (Lemak, 2012). Mapping of CB1 receptor expression is on-going, but indicates that the eCB system can modulate the release of many stress-related neural signals including: corticotropin releasing factor (CRF), CORT, NE, dopamine (DA), serotonin (5-HT),

glutamate, acetylcholine (ACh), gamma-aminobuteric acid (GABA), and histamine (Freund & Hájos, 2003; Lemak, 2012; López-Moreno, González-Cuevas, Moreno, & Navarro, 2008) in limbic and neuroendocrine structures involved in psychological and physical responses to stress (Herkenham et al., 1991; Herman et al., 2005). The ubiquitous expression of this system and known involvement in negative feedback of neurotransmitter activity that would be increased during acute responding to stress support a role for eCB signaling in limiting the initiation, magnitude and duration of stress-related psychological and physical reactions. Accordingly, the eCB system has been demonstrated to act as a buffering mechanism of stress-induced reactivity in multiple structures and responses that are modulated by acute and repeated stress (Hill, Hellemans, Verma, Gorzalka, & Weinberg, 2012; Hohmann et al., 2005).

The current research is limited, but is largely in agreement with a theoretical involvement of widespread eCB signaling as a synaptic negative feedback mechanism that, when normally functioning, acts as a buffer of the increased limbic and neuroendocrine activity that occurs during psychological stress. Interestingly, pretreatment of mice with an inhibitor of anandamide degradation before restraint stress (to mildly increase the levels of anandamide and magnify stress-related eCB activity) is reported to reduce the phasic HPA axis activation compared to controls (Patel, 2004a). Microinjection of the same indirect anandamide agonist into the basolateral amygdala (BLA) is reported to reduce the acute HPA axis response to restraint stress in rats (Hill et al., 2009b) and diminish anxiety-related behavior in the elevated plus maze in mice (Patel & Hillard, 2006). Collective evidence supports a role of eCB signaling in

regulating stress-induced reactivity in limbic and neuroendocrine structures responsible for psychological, physical, and behavioral responses that are altered in habituation and sensitization.

A limited amount of research demonstrates that repeated stress can alter eCB signaling bidirectionally, in a pattern that supports involvement in habituation and sensitization processes. Multiple rodent stressor paradigms have been reported to result in decreased eCB functionality, ligand and receptor expression in a variety of neural structures that can influence reactivity to stress (Hillard, Weinlander, & Stuhr, 2012). Stressors such as acute predator odor, repeated immobilization, chronic prolonged restraint, chronic social defeat stress, and chronic unpredictable stress have been reported to result in reduced measures of eCB activity, including: CB1 receptor expression, binding and functioning, and tissue levels of anandamide (Campos, Ferreira, da Silva, & Guimarães, 2013; Hill, Carrier, McLaughlin, et al., 2008b; Hill, Hunter, & McEwen, 2009a; Rossi et al., 2008; Wamsteeker, Kuzmiski, & Bains, 2010). Structures reported to display these stress-induced disruptions of the eCB system include: prefrontal cortex (PFC), amygdala, hypothalamus/paraventricular nucleus (PVN), hippocampus, and striatum/nucleus accumbens (see previous refs). Though they were not identified as such, the higher intensity stressors used in these studies have also been reported to result in resistance to habituation and development of sensitization. Considering evidence supporting a role of eCB signaling in buffering stress-reactivity in a cooperative multi-structured manner, the results of these studies

support a possibility that the development of a state of sensitization to stress can be explained by widespread decreases in eCB system activity.

An idea that the development of sensitivity to stress relates to increased wear and tear from repeated physical reactions to habituation-resistant stressors is supported by reports that chronic glucocorticoid treatments decrease CB1 receptors, receptor binding, and eCB ligands in various limbic structures that regulate physical and psychological reactions to stress (Bowles et al., 2012; Hill, Carrier, Ho, et al., 2008a). Further evidence to support eCB system deficiencies in impaired ability to habituate to stress and states of hypersensitivity to stress includes recent reports of eCB deficiencies in human populations with diagnoses of emotional disorders that have been associated with this dysfunctional pattern of responding to stress. Several studies have reported reduced serum eCB ligands (2AG and anandamide) in human disorders of anxiety, depression, and PTSD (Hill, Miller, Carrier, Gorzalka, & Hillard, 2009c; Hill, Miller, Ho, Gorzalka, & Hillard, 2008c; Hillard et al., 2012). This measure has been found to equate to concentrations in tissue (Caillé, Alvarez-Jaimes, Polis, Stouffer, & Parsons, 2007). A postmortem study found decreased CB1 receptors on glial cells in the anterior cingulate of subjects diagnosed with major depression (Koethe et al., 2007). Also, PET imaging in a PTSD patient population was recently used to reveal reduced CB1 receptor activity with a sex-dependent pattern that correlates with differences in prevalence and symptom expression (Bailey, Cordell, Sobin, & Neumeister, 2013; Neumeister, 2012). A common structural pattern in these and other emotional disorders is reduced activity in the PFC and increased activity in the amygdala, which coincides

with behavioral and cognitive hypofrontality and increased emotionality (Herman et al., 2005; McEwen, 2006; Neumeister, 2012; SHIN, 2006; Shin & Liberzon, 2010). This pattern is paralleled by chronically stressed rodents and non-stressed CB1 receptor knockout mice as frontal cortex atrophy (pyramidal neuron dendritic simplification) and amygdala hypertrophy (increased pyramidal neuron dendritic arborization), further supporting a role of eCB system disruption in stress-related emotional disorders (Hill, Hillard, & McEwen, 2011; McEwen, 2006).

Though higher intensity laboratory stressors have been observed to result in disruption of eCB signaling, there are a few reports of repeated stress resulting in increases in eCB levels in limbic and neuroendocrine structures. Though the structural basis of habituation to stress is still unknown, facilitation of inhibitory eCB activity would theoretically be able to mediate the reductions in multiple stress-induced responses that occur during habituation to stress. The eCB ligand 2AG was elevated in mouse hypothalamic tissue after 5 days of restraint stress (Patel, 2004b) as well as BLA tissue after 9 and 10 days of repeated restraint (Hill, McLaughlin, et al., 2010a) (rats); (Patel, Kingsley, Mackie, Marnett, & Winder, 2009) (mice), which associated with significant reduction of HPA axis activity compared to initial restraint. Though these results are sparse, they do demonstrate that milder, habituating stressor regimens can increase eCB activity in structures that result in cannabinoid system decreases in more intense stressor paradigms. Worth noting is that all three of these studies measured eCB content in tissue that was harvested at time points (after 30, 30, 20 minutes of stress) that may have missed the robust peak of stressor-stimulated increases in 2AG and

anandamide that would be expected in based on actions of this system and measures of eCB activity in the periaqueductal grey (Hohmann et al., 2005); stress-induced analgesia, 7-15 min after stimulation). The sustained elevations in eCB ligands in the BLA and hypothalamus may be artifacts of earlier, larger increases, but they may also be related to more general functions than inhibition of the initiation of responding to a familiar stimulus. Current habituation literature is divided on involvement of the BLA in habituation to stress (Carter, Pinnock, & Herbert, 2004), but increased hypothalamic eCB activity could relate to inhibition of HPA axis activation (Wamsteeker et al., 2010).

Chapter 2

Cannabinoid receptor type 1 antagonism significantly modulates basal and loud noise induced neural and HPA axis responses in male Sprague Dawley rats

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Abstract

Altered regulation of the hypothalamic-pituitary-adrenal (HPA) axis is associated with stress-induced changes in cognitive, emotional, and physical health. Recent evidence indicates that the endogenous cannabinoid (eCB) system may modulate HPA axis function both directly and more centrally, via regulation of limbic brain systems that control HPA axis activity. The current study examines the contribution of cannabinoid type 1 (CB1) receptor modulation throughout the neuraxis on control and stress-induced HPA axis activity. Adult male Sprague Dawley rats were given intraperitoneal injections of either CB1 receptor antagonist (AM251, 2mg/kg) or vehicle 30 minutes prior to a session of loud white noise stress (95 dBA for 30 min) or placement in familiar sound-proof chamber. Immediately following stress and control treatments, rats were killed, the brains and pituitary glands were excised for subsequent immediate early gene (c-*fos* mRNA) measurement, and trunk blood was collected for subsequent determination of corticosterone (CORT) and adrenocorticotropic (ACTH) hormone levels. AM251 treatment resulted in a potentiated plasma ACTH response to loud noise stress. AM251 treatment also increased stress-induced plasma CORT levels, but that increase may be due to an increase in basal plasma CORT levels, as was evident in control rats. AM251 treatment produced three distinctive c-*fos* mRNA response patterns across the various brain regions examined. In cortical (prelimbic, infralimbic, somatosensory, and auditory) and some subcortical structures (basolateral amygdala and paraventricular nucleus of the hypothalamus), AM251 treatment produced a substantial increase in c-*fos* mRNA that was comparable to the elevated c-*fos* mRNA levels present in those brain regions

of both vehicle and AM251-treated stressed rats. In some other subcortical structures (bed nucleus of the stria terminalis and medial preoptic area) and the anterior pituitary, AM251 treatment produced a c-*fos* mRNA response pattern that was similar to the response pattern of ACTH hormone levels, i.e. no effect on no noise control levels, but an augmentation of stress-induced levels. Conversely, in the medial geniculate and ventral posterior thalamus, AM251 treatment inhibited stress-induced c-*fos* mRNA induction. These data indicate that disruption of eCB signaling through CB1 receptors results in potentiated neural and endocrine responses to loud noise stress, but also substantial increases in activity in various brain regions and the adrenal gland.

Introduction

Accumulating evidence implicates the endogenous cannabinoid (eCB) system as an important regulator of emotionality, and stress reactivity (Finn, 2010; Gorzalka and Hill, 2009; Hill et al., 2010; Lutz, 2009; Riebe and Wotjak, 2011; Valverde, 2005). eCB modulate central nervous system activity through two established ligands, anandamide (Devane et al., 1992) and 2-arachidonyl glycerol (2AG) (Sugiura et al., 1995), which are rapidly synthesized by specific enzymes in postsynaptic neurons in response to calcium-dependen synaptic signaling or other metabotropic receptor activation (Stella and Piomelli, 2001). Once produced, the highly lipid-soluble eCB interact with presynaptic eCB receptors and downstream second-messenger cascades, where they generally have been shown to inhibit glutamate, GABA, acetylcholine, norepinephrine, and serotonin release, among others (Freund et al., 2003, Schlicker and Kathmann, 2001). Both eCB ligands bind to type 1cannabinoid receptors (CB1), which are the most widely expressed eCB receptors in the central nervous system (Herkenham et al., 1991), and to the centrally more restricted type 2 receptors (CB2) (Atwood and Mackie, 2010). In addition to their central nervous system actions, eCB also have well characterized peripheral actions through the same receptor subtypes (Atwood and Mackie, 2010). Given the widespread influence of eCB on neurotransmission, the overall contribution of eCB activity on specific neural functions has been difficult to precisely define.

Recent studies suggest that the endogenous cannabinoids negatively regulate stress responsiveness (Cota, 2008; Hill and McEwen 2010, Hill et al., 2009; Patel et al.,

2004,2005; Tasker 2004). For instance, genetic deletion of CB1 receptors in mice results in hyperactive hypothalamo-pituitary-adrenal (HPA) axis responses to a variety of laboratory stressors, as indexed by increased plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels (Aso et al., 2008; Cota et al., 2007; Haller et al., 2004; Steiner et al., 2008; Uriguen et al., 2004). CB1 receptor knockout mice also express a heightened circadian peak of plasma CORT and impaired glucocorticoid negative feedback compared to wildtype mice (Cota et al., 2007). Furthermore, systemic pharmacological antagonism of CB1 receptors in mice potentiates CORT release in response to restraint and forced swim stress (Patel et al., 2004; Steiner et al., 2008) as well as stress-induced neuronal activity (as indexed by Fos) in the paraventricular nucleus of the hypothalamus (Patel et al., 2004), cingulate cortex, lateral septum, and nucleus accumbens (Patel et al., 2005). On the other hand, increasing the availability of anandamide by systemic pharmacologic blockade of the enzyme responsible for its degradation results in reduced CORT release to restraint stress (Patel et al., 2004, 2005).

Much of the reported work examining the role of eCB activity in stress and HPA axis regulation has been performed using mice, though rats have been used in examining the specific involvement of the basolateral amygdala (Hill et al., 2009) and hypothalamic nuclei (Di et al., 2003, 2005a,b, 2009; Evanson et al., 2010; Ginsberg et al., 2010). Increases in regional Fos protein expression in multiple limbic regions were observed in mice after administration of a CB1 receptor antagonist followed by restraint challenge, suggesting that the stress modulatory effects of eCB are not limited to the

HPA axis (Patel et al., 2005). To our knowledge, no published work has examined the effects of acute stress and CB1 receptor antagonism on c-*fos* mRNA expression in rats. Differences in reported patterns of stressor-induced limbic eCB levels in mice (Patel et al., 2004, 2005; Rademacher et al., 2008) compared to rats (Hill et al., 2007, 2009) suggest possible species differences that warrant further investigation of the functional contribution of eCB processes in stress responses in rats. In addition, no studies have explored the possibility that CB1 receptor antagonism modifies sensory processing that might then be reflected in limbic and hypothalamic structures, in response to stress (Patel et al., 2005). The current study was therefore undertaken to assess regional c-*fos* mRNA induction in several sensory, limbic, and hypothalamic nuclei, as well as pituitary (ACTH) and adrenal (CORT) hormone responses to CB1 receptor antagonism on control and acute loud noise exposure. We chose to examine c-*fos* mRNA in contrast to Fos protein because of the rapid induction and transient expression of mRNA after the onset of neuronal signaling. c-*fos* mRNA likely provides a tighter temporal representation than Fos protein of relative neural activity to proximal events immediately preceding brain harvesting.

Materials and Methods

Subjects

Forty-two male Sprague-Dawley rats (Harlan, Indianapolis IN) weighing 275-300 grams upon arrival were used. Animals were housed in polycarbonate tubs containing wood shavings, with wire lids providing rat chow and water ad libitum. Conditions in the animal colony were controlled to constant humidity and temperature, with a 12:12 hour light/dark cycle (lights on at 7:00 am). Testing was performed between 8:30 am and 12:30 pm during the circadian nadir for the HPA axis. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and conformed to the United States of America National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

Acclimation

Animals were allowed two weeks of acclimation to the colony before testing. The first week, animals were housed in groups of four. During the second week of acclimation, rats were individually housed and handled daily, in the colony room, from days one through four. On each of the last three days before testing, rats were transported in their home cages from the colony to the testing room, handled, returned to their home cages, and placed inside individual acoustic chambers (without noise exposure) for thirty minutes. This pre-exposure was intended to familiarize the rats to all of the testing procedures and minimize novelty related responses on the test day.

Drug Treatment

Rats were randomly assigned to one of four groups: Vehicle treated and noise exposed (n=10), Vehicle treated controls (n=10), AM251 treated and noise exposed (n=12), and AM251 treated controls (n=10), in a 2 x 2 balanced design. The CB1 antagonist/inverse agonist AM251 (Ascent Scientific, Princeton, NJ) was used to assess the involvement of the endogenous cannabinoid system on control and acute loud noise exposure. AM251 was dissolved in dimethyl sulfoxide (DMSO), Tween 80, and physiological (0.9%) saline (in a 1:1:8 ratio, respectively). We experienced difficulty in keeping AM251 from precipitating out of solution, so on the testing day, a stir plate was used to maintain suspension, and syringes were loaded immediately prior to dosing. AM-251 treated rats received a single intraperitoneal injection of 2 mg/kg, in injection volumes of 1 ml/kg. This dosage was chosen based on pilot testing in our laboratory suggesting that this dose was adequate to produce enhancement of loud-noise induced HPA axis activity. On the test day, rats received a single intraperitoneal injection of AM251 or a similar volume of vehicle (DMSO/Tween 80/0.9% saline) 30 minutes prior to placement in the acoustic chambers.

Loud Noise Procedures

The acoustic chambers used in this experiment have been described in detail in Day et al. 2009. On the testing day, rats were placed in the acoustic chambers in their home cages thirty minutes after vehicle or AM251 injection. Rats were either kept under quiet “no noise” control conditions (background noise of fans approximately 57 dB SPL -

A scale) or loud noise (95 dB) was turned on immediately and remained on for thirty minutes. Immediately upon noise termination or quiet chamber exposure, rats were removed from the acoustic chambers, sacrificed by decapitation, and trunk blood was collected in chilled EDTA-containing Vacutainer tubes. Brains and pituitary glands were immediately harvested and frozen in -30 to -40^{o} C isopentanes.

Corticosterone Enzyme Linked ImmunoSorbent Assays (ELISA)

The corticoterone assay was performed according to the manufacturer's instructions (AssayDesigns, Ann Arbor, MI) with one modification. Ten microliters of plasma in the standard buffer were placed in a hot water bath (65^{o}C) for one hour instead of using the steroid displacement reagent. Levels were quantified on a BioTek Elx808 microplate reader and calculated against a standard curve generated concurrently.

ACTH Immunoradiometric Assay (IRMA)

Plasma (200 ul) was assayed for levels of ACTH using an ImmunoradiometricAssay kit (Diasorin, Stillwater, MN, USA), according to the manufacturer's instructions. Briefly, the plasma was incubated overnight with a ^{125}I-labelled monoclonal antibody specific for ACTH 1–17, a goat polyclonal antibody specific for ACTH 26–39, and a polystyrene bead coated with a mouse anti-goat antibody. Only ACTH 1–39 in the sample bound both antibodies to form an antibody complex. Beads were washed to remove unbound radioactivity, counted with a gamma

counter, and the concentrations of ACTH determined by comparison with a standard curve generated concurrently. All samples from this study were run in the same assay.

In situ Hybridization

The method for *in situ* hybridization histochemistry has been previously described (Day and Akil, 1996). Briefly, 12 μm sections were cut on a cryostat (Leica model 1850), thaw-mounted on polylysine-coated slides and stored at -80^{o}C. A [^{35}S]- UTP-labeled riboprobe against c-*fos* mRNA (680 mer; courtesy of Dr. T. Curran, St Jude Children's Hospital, Memphis TN) was generated using standard transcription methods. Sections were fixed in 4% paraformaldehyde (1 hour), acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride (10 min.) and dehydrated through graded alcohols. Sections were hybridized overnight at 55^{o}C with a [^{35}S]- UTP-labeled riboprobe diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 2x saline sodium citrate (SSC), 50 mM PBS, pH 7.4, 1x Denhardt's solution, and 0.1 mg/ml yeast tRNA. The following day, sections were treated with RNase A, 200 ug/ml at 37 ^{o}C (1 hour), and washed to a final stringency of 0.1x SSC at 65^{o}C (1 hour). Dehydrated sections were exposed to X-ray film (BioMax MR; Eastman Kodak, Rochester, NY) for structure-appropriate times (1-3 weeks) and the films analyzed as described below. Structures chosen for *c-fos* mRNA analysis include regions with high levels of neuronal activity (as indicated by induction of *c-fos* mRNA) in response to acute loud noise stress (Burow et al., 2005).

Semi-quantitative x-ray film analysis

Levels of c-*fos* mRNA were analyzed by computer-assisted optical densitometry. Anatomical landmarks were based on the white matter distribution of unstained tissue sections, according to a standard rat brain atlas (Paxinos and Watson, 1998). Brain sections were captured digitally (CCD camera, model XC-77; Sony, Tokyo, Japan), and the relative optical density of the x-ray film was determined using Scion Image version 4.0 for PC. A macro was written (Dr. S. Campeau) that enabled signal above background to be determined automatically. For each section, a background sample was taken over an area of white matter, and a signal threshold was calculated as mean gray value of background + 3.5 standard deviation. The section was automatically density sliced at this value, so that only pixels with gray values above these criteria were included in the analysis.

Statistical analyses

SPSS version 18.0 was used to perform statistical analyses (Chicago, Il, USA). Values for plasma CORT and ACTH as well as c-*fos* mRNA expression were analyzed using a 2-way analysis of variance (ANOVA), with stress treatment (no noise vs. loud noise) and drug treatment (vehicle vs. AM251) as fixed factors. Significance for all tests was established at a $P = 0.05$. All data presented in the figures are listed as mean values +/- standard error. Outlier values in the data set were identified as those being greater than 2 standard deviations from the mean when included in the data set, and were excluded.

Results

Endocrine responses

Analysis of plasma ACTH revealed significant main effects of stress ($F_{1/34} = 6.59$, $p < 0.05$) and AM251 drug treatment ($F_{1/34} = 35.7$, $p < 0.01$), as well as a significant interaction between drug and stress ($F_{1/34} = 5.46$, $p < 0.05$; Fig. 2.1), indicating that CB1 receptor antagonism by AM251 treatment significantly potentiated the noise stress-induced increase in ACTH. Analysis of plasma CORT from trunk blood taken immediately after sacrifice revealed significant main effects of loud noise stress exposure ($F_{1/37} = 217$, $p < 0.01$) and AM251 drug treatment ($F_{1/37} = 16.8$, $p < 0.01$), but not a significant interaction between stress and drug treatment ($F_{1/37} = .29$, $p = 0.6$; Fig. 2.1). These statistical results indicated that both loud noise exposure and AM251 treatment significantly increased levels of plasma CORT.

c-*fos* mRNA expression

Anterior Pituitary Gland

Anterior Pituitary Gland: Analysis of c-*fos* mRNA expression in the anterior pituitary glands revealed that there were significant main effects of loud noise stress exposure ($F_{1/33} = 320$, $p < 0.01$) and AM251 drug treatment ($F_{1/33} = 7.8$, $p = 0.01$), as well as a significant interaction between loud noise exposure and antagonism of CB1

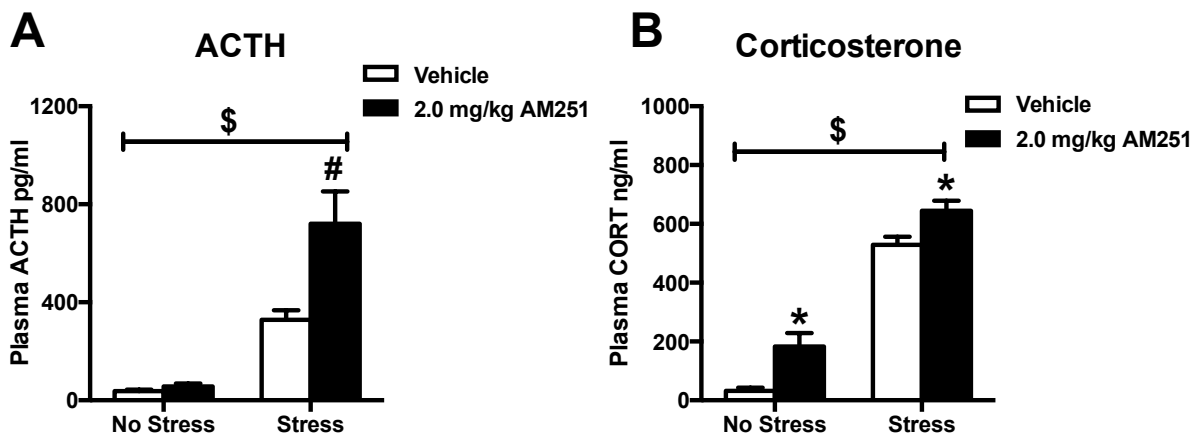

Figure 2.1. Plasma ACTH and corticosterone levels in response to AM251 and stress treatment. **A.** AM251 treatment potentiated the noise stress-induced increase in plasma ACTH. Significant main effects of loud noise stress ($, $p < 0.05$); and significant interaction of loud noise stress and AM251 treatment (#, $p < 0.05$). **B.** AM251 treatment and loud noise stress increase circulating corticosterone levels. Significant main effects of loud noise stress ($, $p < 0.001$), and AM251 drug treatment (*, $p < 0.001$).

receptors with AM251 ($F_{1/33} = 6.4$, $p < 0.05$ Fig. 2.2). Taken together, these results indicated that systemic AM251 treatment resulted in a potentiation of c-*fos* mRNA expression, but only in response to loud noise stress exposure in anterior pituitary gland tissue. This pattern is similar to the potentiation of response measured in plasma ACTH by interaction of loud noise exposure and AM251. See Figure 2.3 for representative autoradiographs of pituitary gland c-*fos* mRNA. Intermediate and posterior lobes of pituitary glands were not quantified, but are referred to in Figure 2.3 for perspective.

Paraventricular Nucleus of the Hypothalamus

Expression of c-*fos* mRNA in the paraventricular hypothalamic nucleus (PVN) was increased by AM251 compared to vehicle-treated controls, but was not significantly

potentiated by AM251 in the noise stress condition (Fig. 2.2). Significant main effects of loud noise stress ($F_{1/37} = 53.3$, $p < 0.01$) and AM251 treatment ($F_{1/37} = 18.9$, $p <$

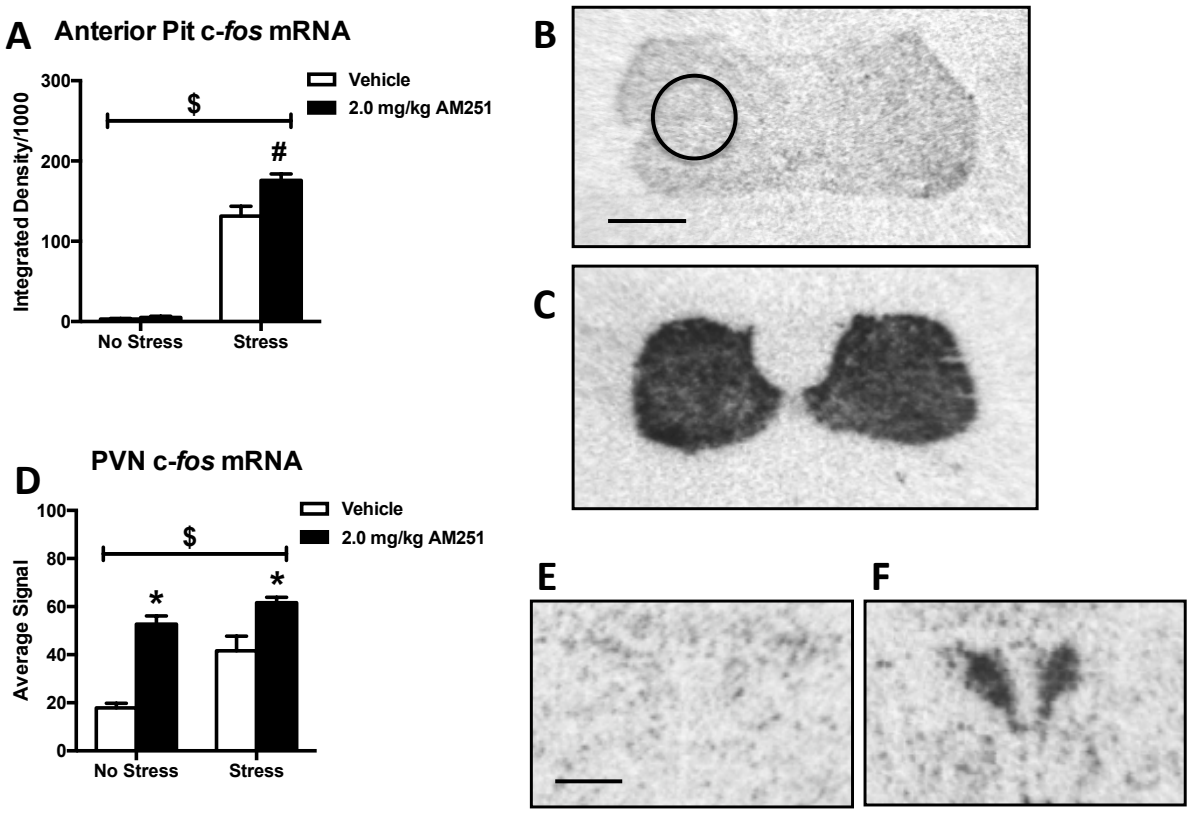

Figure 2.2. ***c-fos*** **mRNA induction in structures intrinsic to the HPA axis. A.** AM251 treatment significantly increased loud noise stress-induced c-*fos* mRNA in the anterior pituitary gland (#, $p < 0.05$). Significant main effects of loud noise stress (\$, $p < 0.001$) **B.** Representative autoradiograph of *c-fos* mRNA expression in the anterior pituitary gland of a rat in no-noise condition, with quantified region of interest marked by a circle. Scale bar = 1 mm. **C.** Representative pituitary gland autoradiograph from a rat exposed to loud noise stress. **D.** AM251 (*, $p < 0.001$) and loud noise stress (\$, $p < 0.001$) significantly induced *c-fos* mRNA expression in the paraventricular nucleus of the hypothalamus. **E.** Representative autoradiograph of c-*fos* mRNA expression in the paraventricular nucleus of a rat in the no-noise condition. Scale bar = 800 µm. **F.** Representative autoradiograph of c-*fos* mRNA expression of the paraventricular nucleus of a rat in the loud noise stress condition.

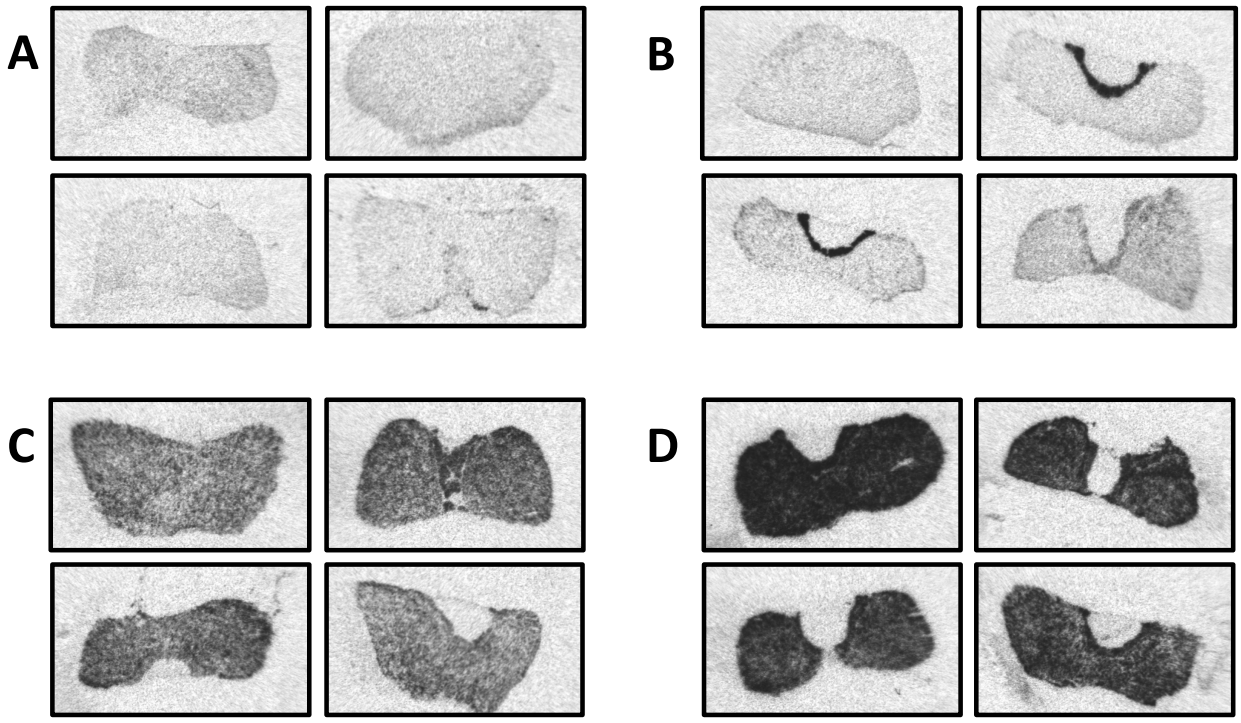

Figure 2.3 Representative autoradiographs showing effects of CB1 receptor antagonism and acute loud noise stress on pituitary gland c-*fos* mRNA. A. Vehicle-treated, Non-stressed; **B.** AM251-treated, Non-stressed; **C.** Vehicle-treated, Acute Stress; **D.** AM251-treated, Acute stress. In distinct contrast to the pattern of PVN level c-*fos* mRNA induction, anterior pituitary gland c-*fos* mRNA induction displays an interaction of CB1 receptor antagonism and acute loud noise stress that is visible as potentiated stress-induced HPA axis response. Two pictures of AM251-treated, non-stressed (B) pituitary glands show a dark expression in the intermediate lobe, which was visible in many of the pituitary glands from this treatment group. This was unexpected, but is confirmation of successful drug treatment, and indicates a disruption of CB1 receptor-mediated tonic inhibition of activity in this region or in the PVN that is not a tonic inhibition of anterior pituitary gland tissue. Each picture in this figure is an example from an individual rat. Due to the size and relative fragility, pituitary glands were cut from various angles, depending on how they were frozen on testing day. Therefore, intermediate and posterior lobe is not always visible.

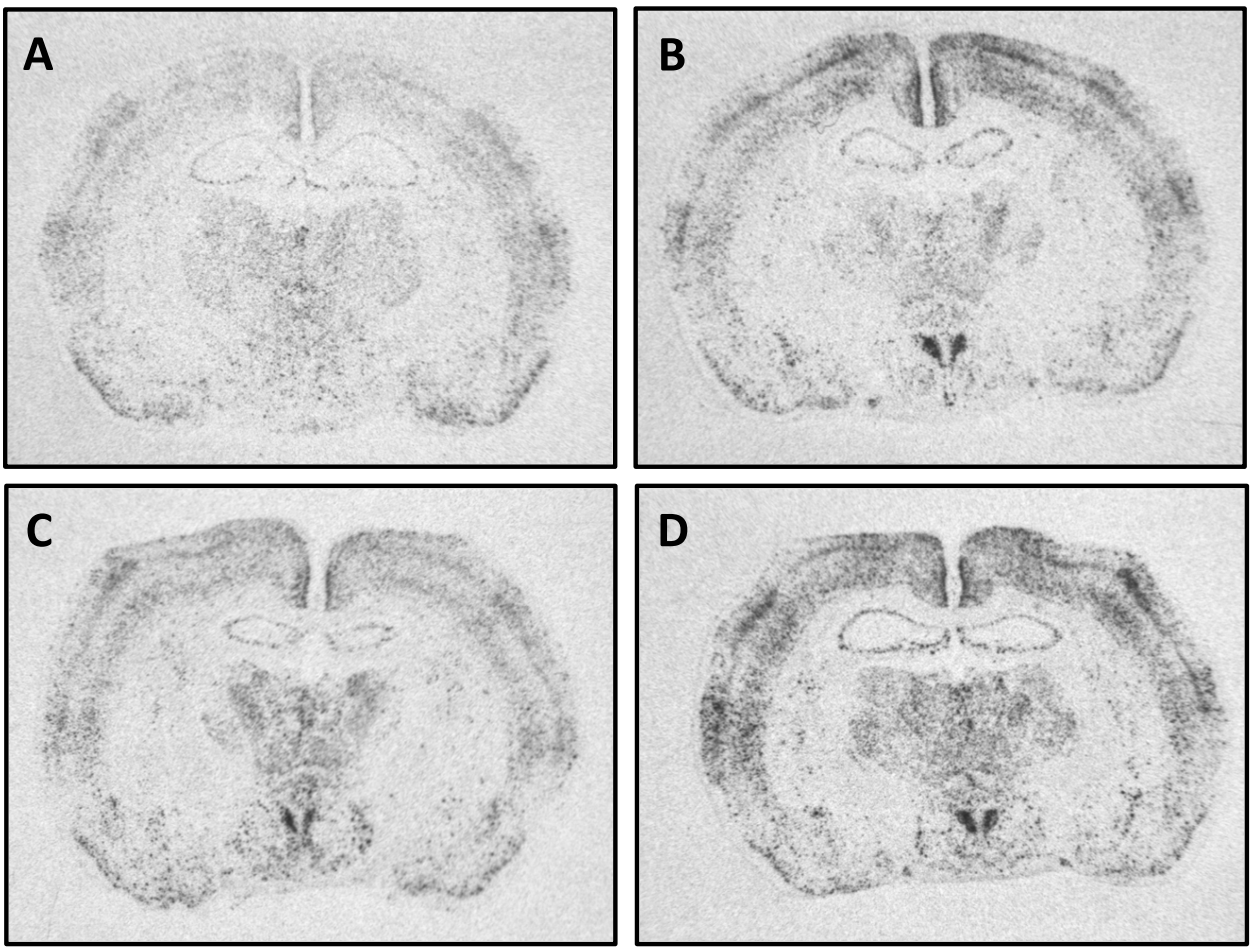

Figure 2.4 Representative autoradiographs showing effects of CB1 receptor antagonism and acute loud noise stress on PVN-level c-*fos* mRNA. A. Vehicle-treated, Non-stressed; **B.** AM251-treated, Non-stressed; **C.** Vehicle-treated, Acute stress; **D.** AM251-treated, Acute stress. CB1 receptor antagonism with AM251 increases c-*fos* mRNA in the cortex, amygdala, and paraventricular nucleus of the hypothalamus (PVN). The AM251-evoked increase in neural activity in these regions is similar to the stress-evoked increases in vehicle-treated rats. Interaction of CB1 receptor antagonism and loud noise stress on c-*fos* mRNA induction is not visible in these regions, though AM251-treated, acutely stressed rats display the highest level of induction in all three of these regions.

0.01) indicated that both loud noise stress and AM251 treatment significantly increased c-*fos* mRNA expression in the PVN. Analysis revealed a near-significant trend of interaction between loud noise stress exposure and AM251 treatment in c-*fos* mRNA expression ($F_{1/37} = 3.91$, $p = 0.06$). It should be noted that several film exposure times were tested to minimize a possible ceiling effect of the radionuclide exposure on the film, especially in the AM251/loud noise condition. In contrast to the other areas quantified using an appropriately shaped template, each PVN section was individually traced, reflecting the normal variance in shape and size of this region. We report the average signal of this structure rather than the integrated density, which is importantly influenced by the exact measurement area. See Figure 2.4 for representative autoradiographs of c-*fos* mRNA expression in the PVN.

Stress-reactive sub-cortical brain structures

Expression of c-*fos* mRNA in additional forebrain and midbrain structures revealed significant increases in response to loud noise stress in all areas quantified as compared to vehicle-treated rats. See Figure 2.5 for diagrammatic representations of the geometric shapes used as templates for quantification, which are placed in the exact regions analyzed. Antagonism of CB1 receptors by systemic administration of AM251 resulted in three distinct patterns of c-*fos* mRNA induction: 1- Significant potentiation of stress-induced c-*fos* mRNA induction without reliable increases above control effects; 2- Significant potentiation of c-*fos* mRNA induction in the control condition without further increase following noise stress; and 3- Significant reduction in c-*fos* mRNA induction by

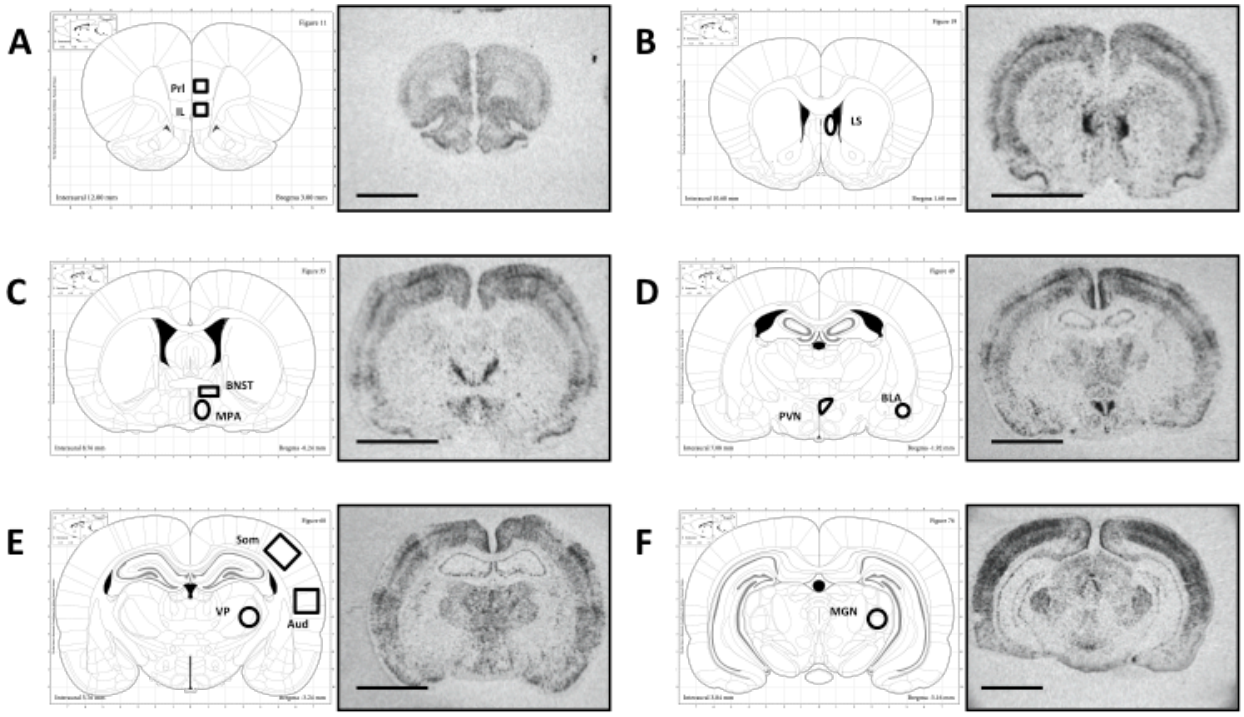

Figure 2.5 Diagrammatic representations from the Paxinos and Watson (1998) atlas of brain sections with quantified regions of interest marked by thick geometric figures. Scale bars in each section = 5 mm. **A.** Prl: prelimbic cortex, IL: infralimbic cortex. **B.** LS: lateral septum. **C.** BSTav: anteroventral bed nucleus of the stria terminalis, MPA: medial preoptic area of the hypothalamus. **D.** PVN: paraventricular nucleus of the hypothalamus (each section individually traced), BLA: basolateral nucleus of the amygdala. **E.** Som: somatosensory cortex, Aud: auditory cortex, VP: ventral posterior nucleus of the thalamus. **F.** MGN: medial geniculate nucleus of the thalamus.

AM251 treatment, especially in the noise stress condition. Structures found to display significant potentiation of stress-induced c-*fos* mRNA induction by AM251 included the anteroventral bed nucleus of the stria terminalis (BSTav) ($F_{1/38} = 5.69$, $p < 0.01$) and medial preoptic area of the hypothalamus (MPA) ($F_{1/37} = 6.31$, $p < 0.05$) as measured by significant interactions between loud noise exposure and AM251 drug treatment (Fig. 2.6). See Figure 2.7 for representative autoradiographs of c-*fos* mRNA in the BSTav.

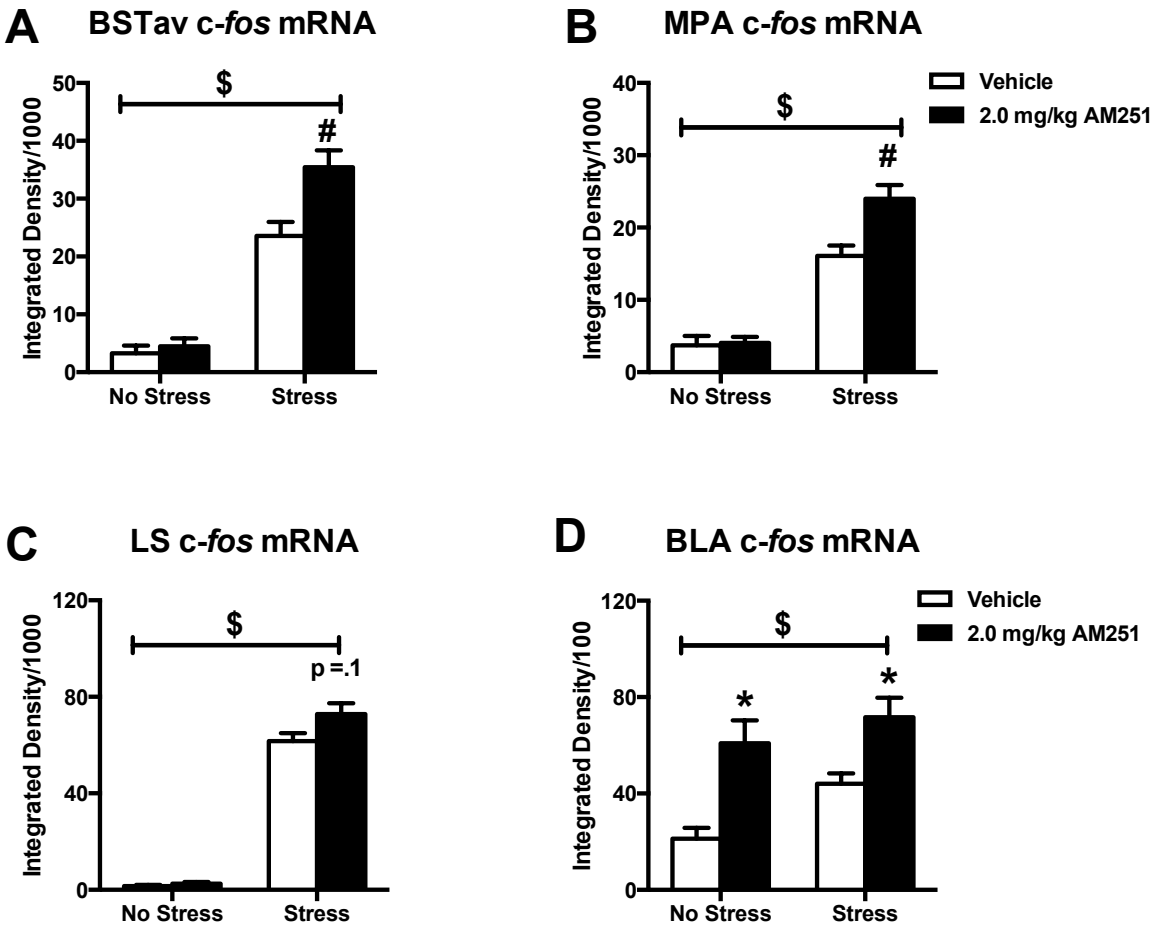

Figure 2.6 ***c-fos*** **mRNA induction in stress-reactive subcortical brain structures.** AM251 drug treatment significantly potentiated loud noise stress-induced neural activity in the anteroventral bed nucleus of the stria terminalis and medial preoptic area. Anteroventral bed nucleus of the stria terminalis: Significant main effects of loud noise stress ($, $p < 0.01$), and significant interaction of noise stress and AM251 drug treatment (#, $p < 0.05$). Medial preoptic area: Significant main effects of loud noise stress ($, $p = 0.01$), and significant interaction of noise stress and AM251 drug treatment (#, $p < 0.05$). Lateral septum: Significant main effects of loud noise stress ($, $p < 0.001$), but no significant interaction between noise stress and AM251 treatment ($p = 0.10$). Basolateral amygdala: Significant main effects of loud noise stress ($, $p < 0.05$), AM251 drug treatment (*, $p < 0.001$), but no significant interaction of noise stress and AM251 drug treatment ($p = 0.411$).

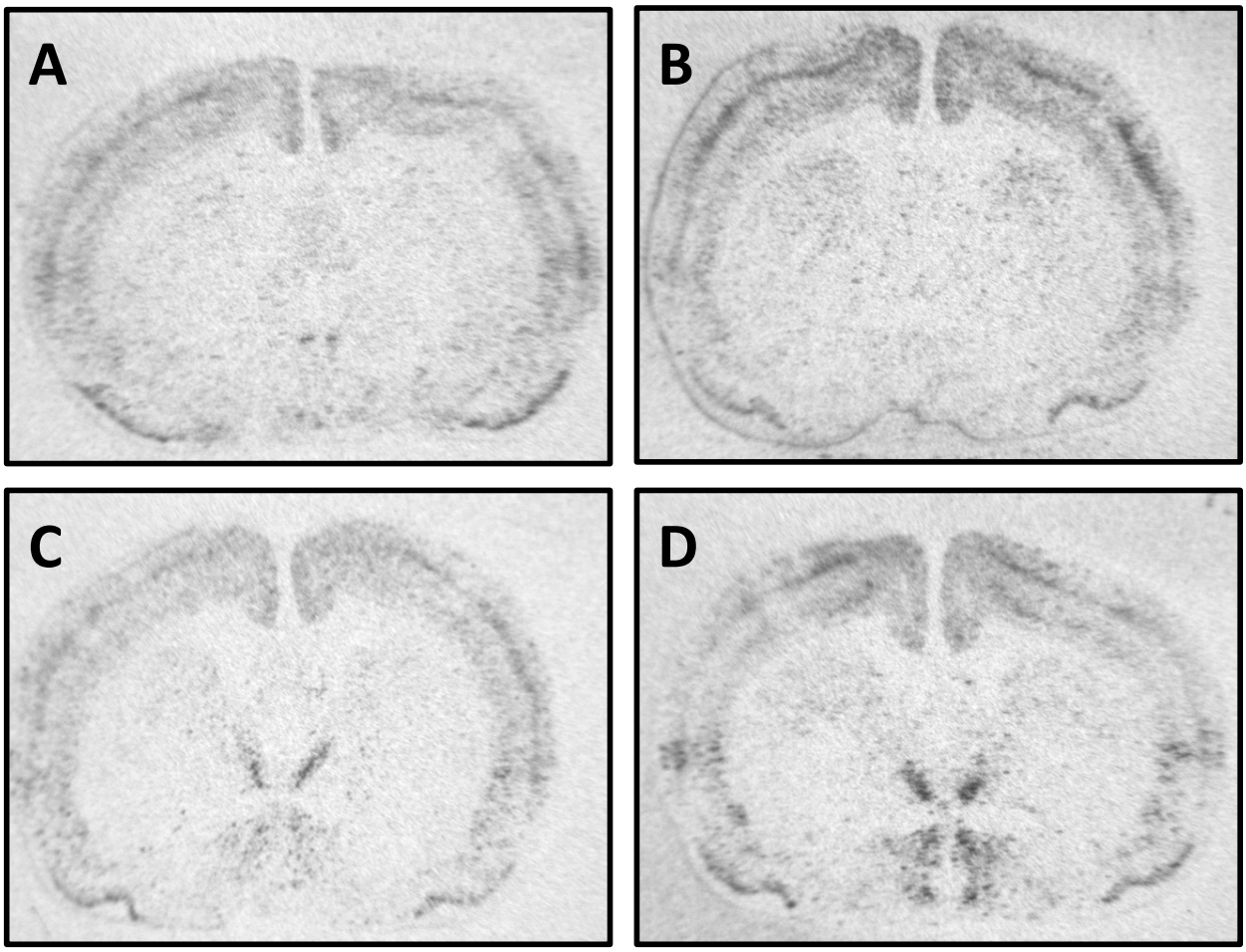

Figure 2.7 Representative autoradiographs showing effects of CB1 receptor antagonism and acute loud noise stress on c-*fos* mRNA induction in the anteroventral bed nucleus of the stria terminalis (BNSTav) and medial preoptic area (MPA) A. Vehicle-treated, Non-stressed; **B.** AM251-treated, Non-stressed; **C.** Vehicle-treated, Acute Stress; **D.** AM251-treated, Acute stress. CB1 receptor antagonism was measured to potentiate loud noise stress-evoked c-*fos* mRNA induction in the BSTav and MPA. Lack of c-*fos* mRNA induction in AM251-treated, non-stressed rats in these regions indicates a lack of constitutive CB1 receptor-involving tonic inhibition in these regions.

Though statistically insignificant, the interaction patterns of c-*fos* mRNA expression in the lateral septum ($p = 0.1$) were similar to that of the MPA and BSTAV, with no increase in c-*fos* mRNA resulting from AM251 treatment without noise, but potentiated induction when combined with loud noise stress treatment (see Figure 2.8 for representative autoradiographs of LS c-*fos* mRNA). The ventral lateral septum was

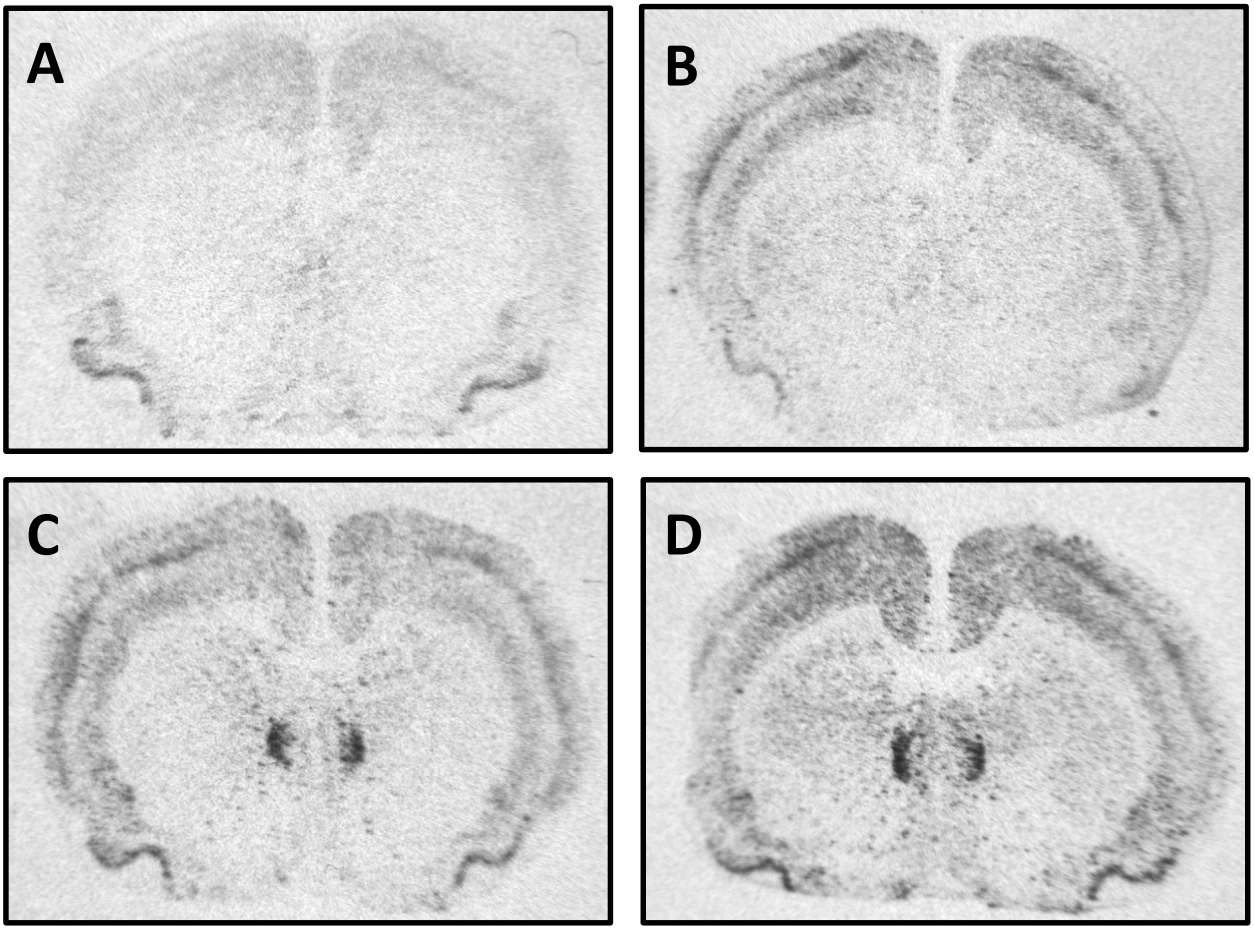

Figure 2.8 Representative autoradiographs showing effects of CB1 receptor antagonism and acute loud noise stress on c-*fos* mRNA induction in the lateral septum (LS). A. Vehicle-treated, Non-stressed; **B.** AM251-treated, Non-stressed; **C.** Vehicle-treated, Acute Stress; **D.** AM251-treated, Acute stress. The lateral septum displays robust c-*fos* mRNA induction in response to acute stress. CB1 receptor antagonism did not increase c-*fos* mRNA induction in non-stressed rats, suggesting that the eCB system is not mediating tonic inhibition of activity in this region. Note the increase in cortex c-*fos* mRNA induction in AM251-treated, non-stressed rats, which was not measured at this level, but is consistent with AM251-evoked stimulation of activity in the frontal cortex and sensory cortical regions. AM251 treatment was measured to trend towards potentiating LS c-*fos* mRNA induction in stressed rats.

quantified separately, and expressed a non-significant ($p > 0.05$) pattern similar to the lateral septum (data not shown). In contrast, c-*fos* mRNA expression in the basolateral amygdala (BLA) was substantially increased by AM251 drug treatment with and without loud noise (Fig. 2.6). Analysis of the BLA revealed significant main effects of loud noise

stress exposure ($F_{1/37} = 5.32$, $p < 0.05$) and AM251 treatment ($F_{1/37} = 21.3$, $p < 0.01$), without significant interaction ($F_{1/37} = 0.69$, $p = 0.41$). See figure 2.4 for representative autoradiographs of BLA c-*fos* mRNA expression at the level of the PVN.

Surprisingly, in both thalamic areas measured, antagonism of CB1 receptors by AM251 treatment resulted in a pattern of inhibition of c-*fos* mRNA expression in stressed rats (Fig. 2.9). This distinct interaction effect was measured in the somatosensory thalamus (VP: combined measure of ventral posterolateral (VPL) and ventral posteromedial (VPM) nuclei) ($F_{1/37} = 6.05$, $p < 0.05$), but a significant interaction was not measured in the auditory medial geniculate nucleus (MGN) ($F_{1/36} = 1.77$, $p = 0.19$, Fig. 2.9).

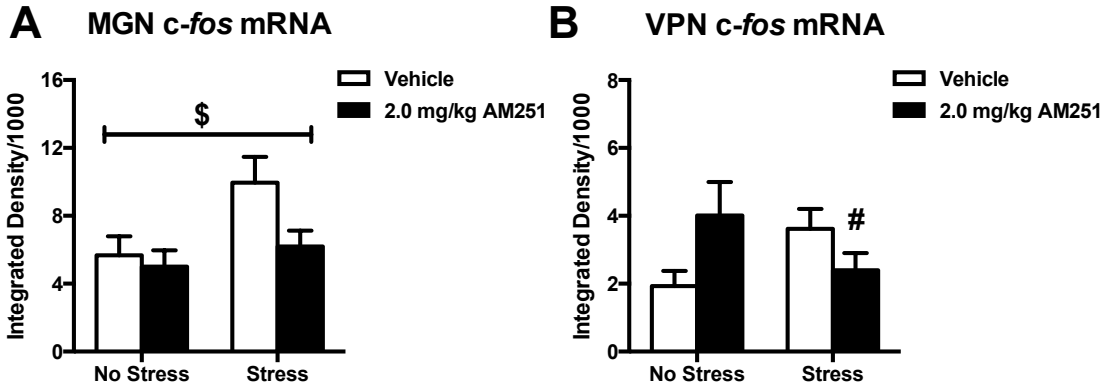

Figure 2.9 ***c-fos*** **mRNA induction in thalamic regions.** A contrasting pattern of interaction of loud noise stress and AM251 drug treatment was measured in both thalamic areas quantified (medial geniculate and ventral posterior nuclei) compared to other limbic areas. Systemic AM251 drug treatment resulted in inhibition of *c-fos* mRNA induction in both nuclei in response to loud noise stress. **A.** Medial geniculate nucleus: Significant effect of loud noise stress ($, $p < 0.05$). **B.** Ventral posterior nucleus: Significant interaction of loud noise stress and AM251 drug treatment (#, $p < 0.05$).

Cortical Areas

Significant main effects of loud noise stress were measured in the auditory ($p < 0.01$), prelimbic ($p < 0.01$), and infralimbic cortices ($p< 0.01$), but not in the somatosensory cortex ($p = 0.1$). AM251 treatment significantly increased c-*fos* mRNA induction in the auditory ($p < 0.01$), somatosensory ($p = 0.01$), and prelimbic cortices ($p< 0.05$),

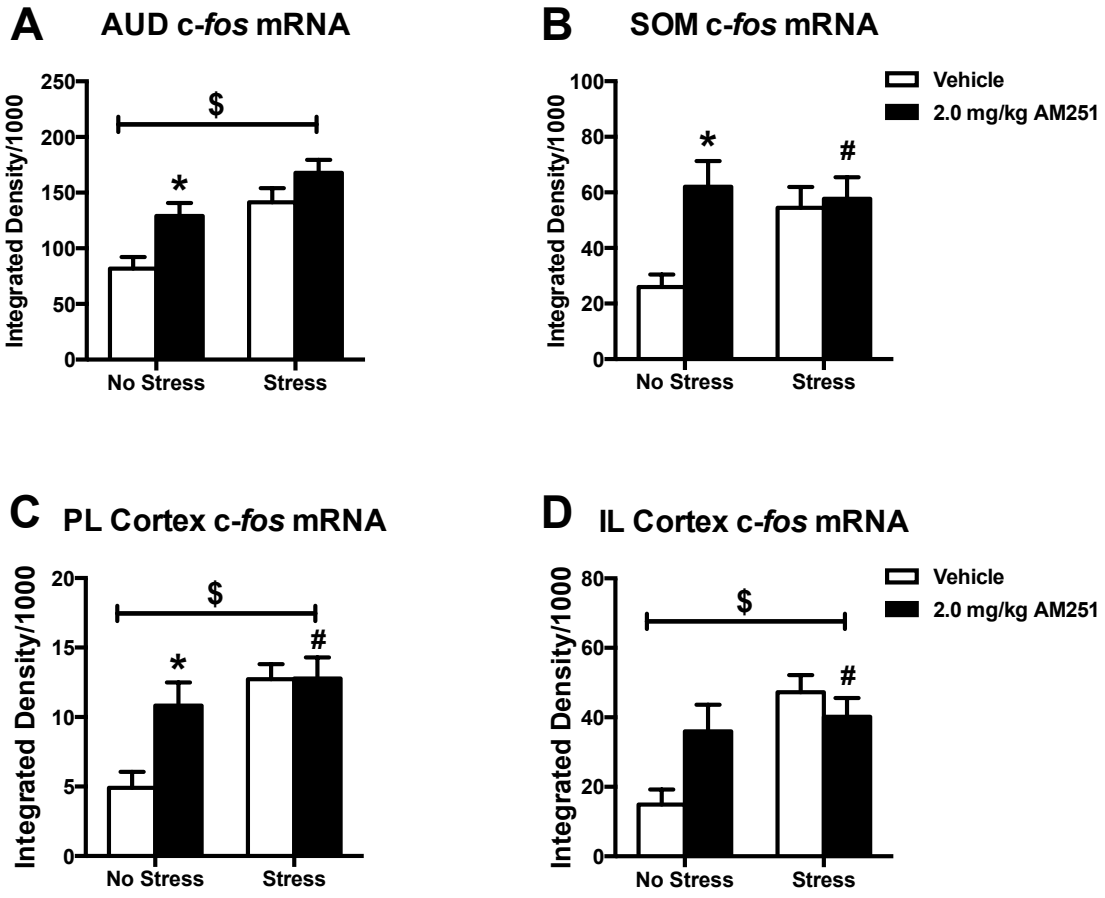

Figure 2.10 ***c-fos*** **mRNA induction patterns in the auditory, somatosensory, prelimbic and infralimbic cortices.** AM251 drug treatment significantly increased *c-fos* mRNA induction in control rats in areas quantified. Significant effects of AM251 drug treatment: (*, Aud: $p < 0.01$, Som: $p < 0.05$, Prl: $p < 0.05$, IL: $p = 0.23$). Significant effects of loud noise stress: ($, Aud: $p < 0.001$, Prl: $p < 0.001$, IL: $p < 0.01$). This response was not further potentiated by AM251 drug treatment, but significant interactions between loud noise exposure and AM251 treatment were measured in the somatosensory cortex (#, $p < 0.05$), prelimbic cortex ($p < 0.05$) and infralimbic cortex ($p < 0.05$).

but not in the infralimbic cortex ($p = 0.23$). The effects of CB1 receptor antagonism on c-*fos* mRNA expression were not significantly potentiated by exposure to loud noise stress in any of these cortical regions (Fig. 2.10). Significant interactions between loud noise stress exposure and AM251 drug treatment were measured in the somatosensory cortex ($F_{1/38} = 4.8$, $p = 0.04$), prelimbic cortex ($F_{1/37} = 4.26$, $p = 0.05$), and infralimbic cortex ($F_{1/37} = 5.89$, $p = 0.02$), but not the auditory cortex ($F_{1/38} = 0.75$, $p = 0.39$). AM251 induced a very similar pattern of c-*fos* mRNA induction in all four of these cortical regions, which may approach a response ceiling in the stress condition or may reflect influence of CB1 receptor antagonism on GABAergic neurons.

Discussion

The results of this study contribute to the knowledge of the involvement of endocannabinoid signaling in the nervous system of male Sprague Dawley rats in several ways. Plasma corticosterone and ACTH were examined as indices of stressor-induced HPA axis activation, and c-*fos* mRNA expression was used as a measure of recent neural activity (Kovacs, 1998). Vehicle treated rats displayed increases in HPA axis activity, and widespread induction of c-*fos* mRNA in response to an acute thirty-minute episode of loud noise exposure, as previously reported (Burow et al., 2005, Campeau and Watson 1997). Antagonism of type 1 eCB receptors by intraperitoneal administration of 2 mg/kg AM251 altered either control and/or stress-induced responses for all measures. In most cases AM251 treatment increased the control or stress-induced levels of HPA axis activity and regional c-*fos* mRNA induction. These results

are consistent with previous reports that disruption of inhibitory eCB signaling increases HPA axis activity in non-stressed and acute stress conditions (Aso et al., 2008; Cota et al., 2007; Haller et al., 2004; Hill and McEwen, 2010; Patel et al., 2004, 2005; Steiner et al., 2008; Uriguen et al., 2004). Interestingly, however, AM251 treatment inhibited stress-induced levels of c-*fos* mRNA in the measured thalamic nuclei. The data suggest that the CB1 receptor antagonist-mediated potentiation of HPA axis response to acute loud noise stress was related to modulation of limbic processing of the stressor. The increases in neural and endocrine activity from administration of AM251 in the no-noise condition may suggest a widespread involvement of eCB signaling in regulating activity in non- or low-stressed conditions. It is important to consider that measures taken from rats administered vehicle or AM251 without noise stress may reflect activity related to stress from the injection procedure, and may not reflect a true “non-stressed” condition. It would be of interest to habituate all rats to the stress of the i.p. injection procedure for several days before the testing day, or administer the drug and vehicle remotely via surgically implanted catheters to eventually answer this question.

Acute antagonism of CB1 receptors in control condition

HPA axis activation

Recent studies have suggested the possibility of tonic inhibitory eCB tone constraining HPA axis output through interactions at the level of the basolateral complex of the amygdala (Hill et al. 2009) and paraventricular hypothalamic nucleus (Di et al. 2009, Hill and McEwen 2010, Patel et al. 2004, 2005). One hour after administration of

AM251, robust induction of c-*fos* mRNA in the basolateral complex of the amygdala and paraventricular hypothalamic nucleus were measured, and also corresponded to an increase in plasma corticosterone. However, this was observed without a significant concurrent elevation of circulating ACTH levels or c-*fos* mRNA induction in the anterior pituitary gland 1 hr after treatment, reducing the possibility that the elevation of basal corticosterone was due to a prolonged effect of AM251 treatment on anterior pituitary-mediated neurohormone secretion. It is possible, however, that there was a transient stimulatory effect of AM251 treatment on basal ACTH secretion which was missed by the 60 minute delay of the measurements, which would also explain the more sustained elevation of corticosterone. If such was the case, then it also raises the possibility that the increased CORT measured in the plasma from AM251-treated control rats reflected potentiation of a putative transient activation of the HPA axis by the mild stress of the i.p. injection procedure, rather than a true increase in adrenocortical basal activity (Galiegue et al., 1995). An alternative explanation for the increase in CORT measured in AM251-treated control rats is disruption of a tonic eCB-mediated inhibition of activity at the level of the adrenal cortex.

CB1 antagonist mediated increases in neural activity

Of interest, significant increases in c-*fos* mRNA were observed in control AM251-treated rats in all cortical areas examined, including the prelimbic, infralimbic, somatosensory, and auditory cortices. Due to generally high CB1 receptor levels in all cortical areas (Herkenham et al., 1991), this observation may reflect generally increased

cortical activity locally due to removal of tonic eCB-mediated cortical inhibition. Alternatively, higher cortical c-*fos* mRNA induction might be secondary to higher levels of sensory-dependent basal thalamic activity, but this possibility seems unlikely because there was no corresponding increase of c-*fos* mRNA in the thalamic medial geniculate and ventroposterior nuclei. Higher basal c-*fos* mRNA expression in the basolateral complex of the amygdala and the paraventricular hypothalamic nucleus after AM251 treatment, may also be due to either local disinhibition of eCB1 receptors, or increased inputs from cortical or other brain regions. Localized administration of AM251 into the basolateral amygdaloid complex or paraventricular hypothalamic nucleus will be necessary to further characterize these effects and to understand specific contribution of eCB signaling in these structures during both drug treatment and acute stress. Again, measures collected from control rats in this experiment may reflect activity related to the mild stress of the i.p. injection procedure, which in the case of AM251-treated control rats, would possibly reflect CB1 antagonist-mediated potentiation of this activity.

The lack of significant potentiation of c-*fos* expression measured in some areas (e.g. cortical areas, BLA, PVN) does not rule out local eCB involvement in acute stress responsiveness. Our measures are taken from a single time point immediately after a 3-minute exposure to loud noise stress. Recent work by Hill et al., (2011) has shown that disruption of eCB activity in the prefrontal cortex leads to a prolongation of HPA axis response to acute stress. It is possible that AM251 could affect the time course of activity in a pattern not detectable at the time of our measurements, which may have been visible if tissue was taken from brains at a later time after the acute stress session.

AM251-mediated potentiation of responses to stress

Antagonism of CB1 receptors with AM251 resulted in potentiation of the acute HPA axis response to a thirty-minute presentation of loud noise stress, as measured by plasma ACTH. The high levels of circulating plasma corticosterone obtained with loud noise exposure might have precluded a clear observation of AM251-induced potentiation of CORT levels. Analysis of c-*fos* mRNA induction in anterior pituitary gland tissue supported this pattern of potentiation. The increased response to loud noise did not appear to be explained by AM251-mediated potentiation of activity specifically at the level of the paraventricular nucleus of the hypothalamus, although a ceiling effect cannot be ruled out. This conclusion is consistent with the finding by Evanson et al. (2010) that microinfusion of AM251 into the paraventricular nucleus of the hypothalamus did not alter the HPA axis response to acute stress challenge. Significant AM251-induced potentiation of c-*fos* mRNA responses to loud noise stress was only observed in some of the limbic regions known to project to the paraventricular nucleus of the hypothalamus, including the anterior bed nucleus of the stria terminalis (anteroventral nuclei), the medial preoptic area, and a similar trend of induction was measured in the lateral septum. Surprisingly, none of the cortical structures measured displayed potentiated c-*fos* mRNA induction beyond the levels observed to loud noise exposed vehicle-treated rats, perhaps again reflecting a ceiling effect. Importantly, AM251 inhibited stress-induced induction of c-*fos* mRNA in the medial geniculate body, and the ventroposterior thalamic nuclei. This thalamic inhibition could be a result of the elevated

cortical activity in primary sensory cortex that provides feedback on thalamic sensory relay nuclei (Briggs & Usrey, 2007). Regardless of whether the inhibitory effects of AM251 on thalamic neural activity is direct or indirect, a possible consequence is alteration of thalamic processing during loud noise exposure, and perhaps other stress situations, which is a novel finding with regard to eCB signaling and stress interactions. A possible disruption of sensory perception of the stressor may also explain the non-additive effects of AM251 and stress in the cortical regions investigated. The inhibition of activity in the sensory regions of AM251-treated, noise-stressed rats is consistent with the findings of Ho et al., (2010) who reported localized subdural administration of a CB1 receptor antagonist to inhibit activity in the whisker barrel cortex in response to whisker movement. It should be noted that reduction of the medial geniculate body functions by neurochemical lesions or inactivation with muscimol reliably inhibits HPA axis responses to loud noise stress (Campeau et al., 1997, Day et al., 2009). The prediction, from these observations, would be that the AM251- mediated reduction in c-*fos* mRNA induction during loud noise exposure in the auditory thalamus would reduce HPA axis responses. At the very least, these results minimize the possibility that AM251 was potentiating HPA axis activity and c-*fos* mRNA induction in several limbic regions by simply potentiating activity in subcortical sensory processing regions, as AM251 induced a reduction in loud noise-induced c-*fos* mRNA levels in thalamic regions, including the medial geniculate body. Taken together, these data further suggest that CB1 antagonism specifically increases HPA axis response to loud noise stress by

potentiating activity in limbic structures projecting to the HPA axis, perhaps together with potentiation of intrinsic HPA axis structures (i.e., anterior pituitary).

It is possible that the increased limbic response to loud noise stress in rats treated with AM251 would be associated with increased emotional and/or cognitive perception of the stimulus as threatening. Increased sensitivity to activation in neuronal systems responsible for cognitive perception or processing of a stimulus as threatening, or in neural structures involved in the subsequent heightening of arousal in response to perceived threat may result in a heightened response to the situation and may lead to disproportionately large psychological and/or physical reaction. Evidence to support a relationship of eCB deficiency to increased psychological response to stressful circumstance was reported in 2004 by Haller et al., who found that CB1 KO mice exhibited increased anxious behaviors in a context-dependent manner.

The increased neural and endocrine responses to loud noise stress measured in rats that were administered AM251 in this study suggest that deficiency in eCB signaling at CB1 receptors would likely lead to increased sensitivity to psychological stress. Increased responsiveness to repeated psychological stressors would likely result in exacerbation of physical and emotional changes resulting from chronic stress. In this way, deficiency in neural eCB signaling may be an initiating factor in the etiology of stress-induced disruption of health.

eCB deficiency may also be involved in the perpetuation of physical and psychological state of increased vulnerability to stress given reports that both chronic restraint stress and chronic glucocorticoid administration result in widespread reductions

in limbic eCB signaling (Hill et al. 2008, 2009b; Gorzalka et al. 2008). Taken together, this evidence suggests that deficiency in neural eCB signaling may be an important aspect in understanding the interrelationship of physical and psychological factors involved in a downward spiral of chronic stress-induced pathology.

Chapter 3.

Remote, "stress free" administration of CB receptor antagonist AM251 via intraperitoneal catheter to male Sprague Dawley rats reveals dose dependent central and peripheral mechanisms contributing to tonic and phasic psychoneuroendocrine regulation

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Abstract

The endogenous cannabinoid system is widely expressed in the brain and body and contributes to inhibitory regulation of the psychoneuroendocrine system. This experiment was designed to distinguish between the contributions of central compared to peripheral CB1 receptor-dependent inhibitory modulation of basal neural and hypothalamic-pituitary-adrenal (HPA) axis activity and modulation of neural and HPA axis responses stimulated by acute loud noise stress. We recently reported that intraperitoneal (i.p) injection of a CB1 receptor antagonist, AM251, resulted in c-*fos* mRNA induction in the basolateral amygdala (BLA), paraventricular nucleus of the hypothalamus (PVN), and the prefrontal cortex (PFC), as well as an elevation in plasma corticosterone (CORT). This was in distinct contrast to a lack of induction in several measures such as c-*fos* mRNA in regions including the lateral septum (LS), bed nucleus of the stria terminalis (BST), medial preoptic area (MPA), anterior pituitary gland, and in plasma adrenocorticotropin hormone (ACTH). This evidence suggested that the endogenous cannabinoid (eCB) system mediates tonic inhibition of activity in some central regions associated with hypothalamic-pituitary-adrenal axis regulation, but peripheral actions at the adrenal glands were not assessed and could not be ruled out. In addition the stress of the intraperitoneal (i.p.) drug injection may have contributed to some of the c-fos mRNA induction observed. To more clearly examine the putative contribution of central vs. peripheral drug effects, and to help minimize potential injection stress effects, adult male Sprague Dawley rats were surgically implanted with i.p. catheters. After recovery from surgery, rats were placed, in their home cages, inside

acoustically-attenuating chambers to acclimate overnight, and their i.p. catheters were connected to a length of PE tubing exteriorized from the chamber. The next morning, at the circadian trough of HPA axis activity, rats were remotely injected with AM251 (1 or 2 mg/kg) or vehicle (n=7-8 per group). Rats were sacrificed 1 hour after administration, and brains, pituitary glands, and adrenal glands were quickly excised and frozen. Results of this study confirm involvement of the eCB system in phasic inhibitory regulation of psychoneuroendocrine stress reactivity and support a role of cooperative regulation by multiple neural regions including the BST, LS, BLA, and PVN, as well as the adrenal cortex. Additionally, remote administration of AM251 itself stimulated c-*fos* mRNA in several brain regions including the BLA, PVN, and auditory cortex (AUD), as well as elevation of plasma CORT. CB1 receptor mRNA was detected in all central and peripheral tissues of interest, and was found to display sensitivity to acute stress and antagonist treatment in bidirectional patterns. These data indicate a multi-faceted role of the eCB system in psychoneuroendocrine regulation that includes constitutive inhibitory tone and activity-dependent phasic inhibition in multiple central and peripheral tissues. Additionally, the results of this study indicate that the mild stress of injection was not a contributing factor to the neural and endocrine activities observed after AM251 injection in our previous study.

Introduction

The contribution of the endogenous cannabinoid (endocannabinoids, eCBs) system to regulation of multiple components of psychoneuroendocrine activity presents a potential difficulty in distinguishing unique components. The eCB system has recently been examined for a contributing role in basal regulation of psychoemotional state and hypothalamic-pituitary-adrenal (HPA) axis activity, and also reactivity to acute and repeated stress (D. P. Finn, 2010; Hill et al., 2012; Hill, McLaughlin, et al., 2010a; Hillard et al., 2012; Lutz, 2009; Patel & Hillard, 2008; Riebe & Wotjak, 2011; Valverde, 2005). Given the widespread presence of eCB ligands and CB1 receptors (Cota, 2007; Herkenham et al., 1990; Lynn & Herkenham, 1994; Mackie, 2008), it is possible that this system simultaneously contributes to inhibitory regulation of activities in a variety of structural components of the psychoneuroendocrine system independently and cooperatively.

We previously reported that systemic pharmacological antagonism of CB1 receptors with AM251 resulted in a potentiation of noise stress-induced neuroendocrine reactivity in rats, as measured by immediate early gene c-*fos* mRNA in multiple limbic neural regions including the anteroventral bed nucleus of the stria terminalis (BSTav) lateral septum (LS), medial preoptic area of the hypothalamus (MPOA), and peripheral measures including the anterior pituitary gland and stress-induced adrenocorticotropic hormone (ACTH) levels (Newsom et al., 2012). These results are in agreement with previous research indicating a multi-structural role of phasic eCB activity in limiting the magnitude of psychoneuroendocrine reactions to psychological stress (D. P. Finn, 2010;

Hill & McEwen, 2010; Lutz, 2009; Riebe & Wotjak, 2011; Valverde, 2005). In the same study, we also found CB1 receptor antagonist treatment in non-stressed controls to result in significant elevation of plasma CORT and robust induction of c-*fos* mRNA in the PVN, basolateral amygdala (BLA), and various prefrontal and sensory cortical regions. These measures support a presence of constitutive activity involving CB1 receptors that mediates tonic inhibition of neural activity in some neural and endocrine tissues. Interestingly, this antagonist-mediated induction of activity appeared to be entirely absent in the neural and endocrine activity measures found to display evidence of potentiated stress-reactivity.

A possible explanation for the increases in activity we measured after AM251 administration was that they were elicited by the stress of the injection procedure, which has been reported to result in minor induction of our stress-reactive measures of interest (Ryabinin et al., 1999) and could be quickly increased or sustained by AM251 (Ginsberg, Pecoraro, Warne, Horneman, & Dallman, 2010). In this explanation, it would still be important to note a distinction between the measures displaying CB1 receptor antagonist-mediated activity from highly stress-reactive regions such as the LS and BSTav (Burow, Day, & Campeau, 2005), which displayed a stark lack of induction of c-*fos* mRNA due to AM251 (Newsom et al., 2012). Another issue relating to this is the apparent incongruence of PVN c-*fos* mRNA induction by AM251 that did not result in anterior pituitary activity, which would be expected to result if the PVN activity indicated whole HPA axis activation. The increase in CORT we measured after AM251 administration indicates the potential that the adrenal gland is under the same type of

local CB1 receptor-dependent inhibitory regulation as the neural regions, which were sensitive to stimulation by AM251 injection. Involvement of CB1 receptors in tonic inhibition of the HPA axis has been suggested, but it is currently unclear which tissues are responsible for this (Cota, 2007). Adrenal and pituitary gland CB1 receptor activity has not been well explored, but should be considered both in interpretation of HPA axis measures as indexes in psychoneuroendocrine research, as well as in therapeutic strategy. Peripheral CB1 receptor antagonists are of recent therapeutic interest in metabolic regulation (Bowles et al., 2015; Di Marzo, Piscitelli, & Mechoulam, 2011). It will be important to determine if this strategy leads to significant elevation of CORT by adrenal or pituitary level activity, which could be useful or detrimental (Sapolsky, 2000). Dose-dependent effects of CB1 receptor antagonist rimonabant (SR141716A) on CORT stimulation have been reported (Patel, 2004a), but it isn't clear whether this activity is due to disruption of central or peripheral CB1 receptor activity (Cota, 2007; Hill & Tasker, 2012; Newsom et al., 2012), or how effects of CB1 receptor inverse agonist and neutral antagonist effects relate to CB1 receptor function (Di, Popescu, & Tasker, 2013; Hill & Tasker, 2012; Ho et al., 2010; Newsom et al., 2012). The current study was designed to more carefully distinguish the contributions of CB1 receptors in inhibitory regulation of basal compared to stress-stimulated, and central compared to peripheral activities in the psychoneuroendocrine system. Additionally, we include two doses of CB1 receptor antagonist AM251 in all comparisons, and remotely administered drug treatment through intraperitoneal (i.p.) catheters to limit the potential confound of injection stress on basal and stress-related measures.

Methods and Materials

Subjects:

Forty-seven male Sprague Dawley rats (Harlan, Indianapolis IN) weighing 275-300 grams upon arrival were used. Animals were housed in polycarbonate tubs containing wood shavings, with wire lids providing rat chow and water ad libitum. Conditions in the animal colony were controlled to constant humidity and temperature, with a 12:12 hour light/dark cycle (lights on at 7:00 am). Testing was performed between 8:00 am and 11:00 am during the circadian nadir for the HPA axis. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and conformed to the United States of America National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

Surgery:

Following a week of acclimation to the colony and daily handling, all rats were surgically implanted with intra-peritoneal (i.p.) catheters to allow for remote administration of drug and vehicle treatments on testing day. This administration method was used to circumvent the potential confounds of handling and injection stress on basal and stress-induced measures. The surgical procedure was performed as in Day and Akil, 1999 and Day et al., 2005, but with a minor alteration. Catheters were externalized at the center of the upper back rather than mounting to the skull. Following

surgery, dust caps were attached to the externalized opening of the catheters, and rats were individually housed. A recovery period of 5-8 days was allowed before each rat's single testing day.

Experimental Design:

Rats were randomly assigned to receive one of three drug treatments (vehicle, 1.0 mg/kg or 2.0 mg/kg AM251) and acute noise stress or no noise control treatment (3x2 factorial design, 7-9 per group) to allow for examination of effects of antagonism of CB1 receptors on basal and stress-induced neural and HPA axis activity. Due to the remote drug administration procedures, 7-8 rats were tested each testing day. Rats were placed in the acoustic chambers overnight to acclimate. Approximately 5:00 pm on the day before testing days, the entire home cage of each rat was placed into acoustically attenuating chambers (described in detail in Day et al., 2009) and a saline-filled length of polyethylene (PE) tubing within a stainless steel flexible connector (Plastics One, Roanoke VA) was connected to the i.p. catheters and exteriorized from the acoustic chamber. Catheter extensions were attached to a fluid swivel that was mounted on additional cage tops to allow for free movement throughout the cage. Water bottles and rat chow were transferred to these cage tops for continued access. Lighting in the acoustic chambers was appropriately controlled to familiar intensity, and a timer was used to maintain light/dark cycle in accordance with the colony schedule. The next morning, during the circadian trough of HPA axis activity, rats were remotely administered AM251 (1.0 or 2.0 mg/kg) or vehicle (Tween80, DMSO, saline at 1:1:8, 1

ml/kg) through the externalized PE tubing using sterile 1 cc syringes via blunted needles. An additional predetermined amount (0.2 ml) of saline was slowly flushed through the tubing following the drug-containing and vehicle solutions to ensure administration of the entire volume. Thirty-minutes after drug administration, rats were exposed to 30 minutes of (95 dB) loud noise stress. Non-stressed control rats remained in the acoustically attenuating chambers for the same amount of time without loud noise exposure (minimal background noise of fans at approximately 57 dB). Treatment initiation was staggered by five-minute intervals to ensure precise standardization of procedure timing. Immediately following cessation of stress treatments, rats were unhooked from their catheters and transported to an adjacent room in which they were rapidly sacrificed by decapitation. Trunk blood was collected in EDTA-coated containers for later quantification of plasma ACTH and CORT. Brains, pituitary glands, and adrenal glands were rapidly excised and frozen for later sectioning and analysis.

Drug Treatment:

The CB1 antagonist/inverse agonist AM251 (Ascent Scientific, Princeton, NJ) was used to assess the involvement of the endogenous cannabinoid system in regulation of plasma CORT and limbic, pituitary and adrenal c-*fos* and CB1 receptor mRNA expression in basal non-stressed conditions and in responses to acute loud noise exposure. AM251 was dissolved in dimethyl sulfoxide (DMSO) upon arrival, and added to Tween 80, and physiological (0.9%) saline (in a 1:1:8 ratio, respectively). Systemic doses of AM251 (2.0 and 1.0 mg/kg) were chosen based on our previous

results demonstrating 2.0 mg/kg to robustly induce plasma CORT increase and neural activity indicated by c-*fos* mRNA induction in several stress-reactive regions of the brain and to potentiate stress-induced increases in ACTH and c-*fos* mRNA in several other regions (Newsom et al., 2012). A lower dose (1.0 mg/kg) was included for examination of possible dose-dependency of these responses.

Corticosterone Enzyme Linked ImmunoSorbent Assays (ELISA)

The corticoterone assay was performed according to the manufacturer's instructions (kit #K014-H5– Arbor Assays, Ann Arbor, MI) using 10 microliters of plasma. Levels were quantified on a BioTek Elx808 microplate reader and calculated against a standard curve generated concurrently.

Adrenocorticotropic Hormone Assay

Plasma (200 ul) was assayed for levels of ACTH using an Immunoradiometric Assay kit (Diasorin, Stillwater, MN, USA), according to the manufacturer's instructions. Briefly, the plasma was incubated overnight with a 125I-labelled monoclonal antibody specific for ACTH 1–17, a goat polyclonal antibody specific for ACTH 26–39, and a polystyrene bead coated with a mouse anti-goat antibody. Only ACTH 1–39 in the sample bound both antibodies to form an antibody complex. Beads were washed to remove unbound radioactivity, counted with a gamma counter, and the concentrations of ACTH determined by comparison with a standard curve generated concurrently. All samples from this study were run in the same assay.

In situ Hybridization

The method for *in situ* hybridization histochemistry has been previously described (Day and Akil, 1996). Briefly, 12 µm sections were cut on a cryostat (Leica model 1850), thaw- mounted on polylysine-coated slides and stored at –80°C. [35S]- UTP-labeled riboprobes against c-*fos* mRNA (680 mer; courtesy of Dr. T. Curran, St Jude Children's Hospital, Memphis TN) and CB1 receptor mRNA (984 mer from the coding region [580-1563] of rat CB1 receptor, NM012784.4 produced by Dr. Heidi Day) were generated using standard transcription methods. Sections were fixed in 4% paraformaldehyde (1 hour), acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride (10 min.) and dehydrated through graded alcohols. Sections were hybridized overnight at 55°C with a [35S]- UTP-labeled riboprobe diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 2× saline sodium citrate (SSC), 50 mM PBS, pH 7.4, 1× Denhardt's solution, and 0.1 mg/ml yeast tRNA. The following day, sections were treated with RNase A, 200 ug/ml at 37 °C (1 hour), and washed to a final stringency of 0.1× SSC at 65°C (1 hour). Dehydrated sections were exposed to X-ray film (BioMax MR; Eastman Kodak, Rochester, NY) for structure-appropriate times (1–3 weeks) and the films analyzed as described below. Specificity of the probes were tested with equivalent sense strands, which produced no hybridization signals in any tests performed.

Semi-quantitative x-ray film analysis

Levels of c-*fos* and CB1 receptor mRNAs were analyzed by computer-assisted optical densitometry. Anatomical landmarks were based on the white matter distribution of unstained tissue sections, according to a standard rat brain atlas (Paxinos and Watson, 1998). Brain sections were captured digitally (CCD camera, model XC-77; Sony, Tokyo, Japan), and the relative optical density of the x-ray film was determined using Scion Image version 4.0 for PC. A macro was written (Dr. S. Campeau) that enabled signal above background to be determined automatically. For each section, a background sample was taken over an area of white matter, and a signal threshold was calculated as mean gray value of background + 3.5 standard deviation. The section was automatically density sliced at this value, so that only pixels with gray values above these criteria were included in averages that were employed in the analysis. Regions of interest were chosen due to results of a previous study demonstrating them to have apparent sensitivity to CB1 receptor antagonism alone or in combination with stress, as well as to better determine peripheral compared to central involvement in psychoneuroendocrine regulation (Newsom et al., 2012). (Regions of interest and quantification templates are in Figure 3.2)

Statistical analyses

Prism (v 6.0, GraphPad Software Inc.) was used for all statistical analyses, which included two-way analyses of variance (ANOVA) for all measures using drug treatment (vehicle, 1.0 mg/kg and 2.0 mg/kg AM251) and stress treatment (acute noise stress, no noise control) as fixed factors. Given that inverse agonist effects of CB1 receptor

antagonists have been reported to be dose-dependent (Patel, 2004a; Trezza et al., 2012), significant ANOVA effects were followed with Fisher's LSD post hoc analyses of all comparisons, for sensitivity. Significance for all tests was established at a $P = 0.05$. All data presented in the figures are listed as mean gray values +/– standard error. Outlier values were identified as those being greater than 2 standard deviations from the group mean when included in the dataset, and were excluded. Additionally, some variation in degrees of freedom reflects sample loss during processing.

Results:

Peripheral HPA axis activity

Plasma CORT and ACTH were measured to assess contribution of eCB signaling to tonic inhibition and stress-reactivity (Figure 3.1). Two-way ANOVA on plasma ACTH values revealed a significant main effect of stress ($F_{(1,38)} = 198.6$, $p < 0.001$) indicating that acute loud noise stress resulted in significant elevation of ACTH in all groups (Fig. 3.1A). There was not a significant main effect of drug ($F_{(2,38)} = 2.86$, $p = 0.07$) or significant interaction ($F_{(2,38)} = 0.77$, $p = 0.47$). Post hoc analyses indicated that 2.0 mg/kg AM251 treatment did not significantly increase plasma ACTH compared to vehicle treatment in non-stressed controls, but this dose did significantly increase stress-induced ACTH compared to vehicle treatment ($p < 0.05$) indicating potentiation of HPA axis stimulation by AM251. However, 1.0 mg/kg AM251 treatment did not increase non-stressed or stress-induced ACTH compared to vehicle treatment. Plasma CORT was found to display a different pattern (Fig. 3.1B). Two-way ANOVA indicated

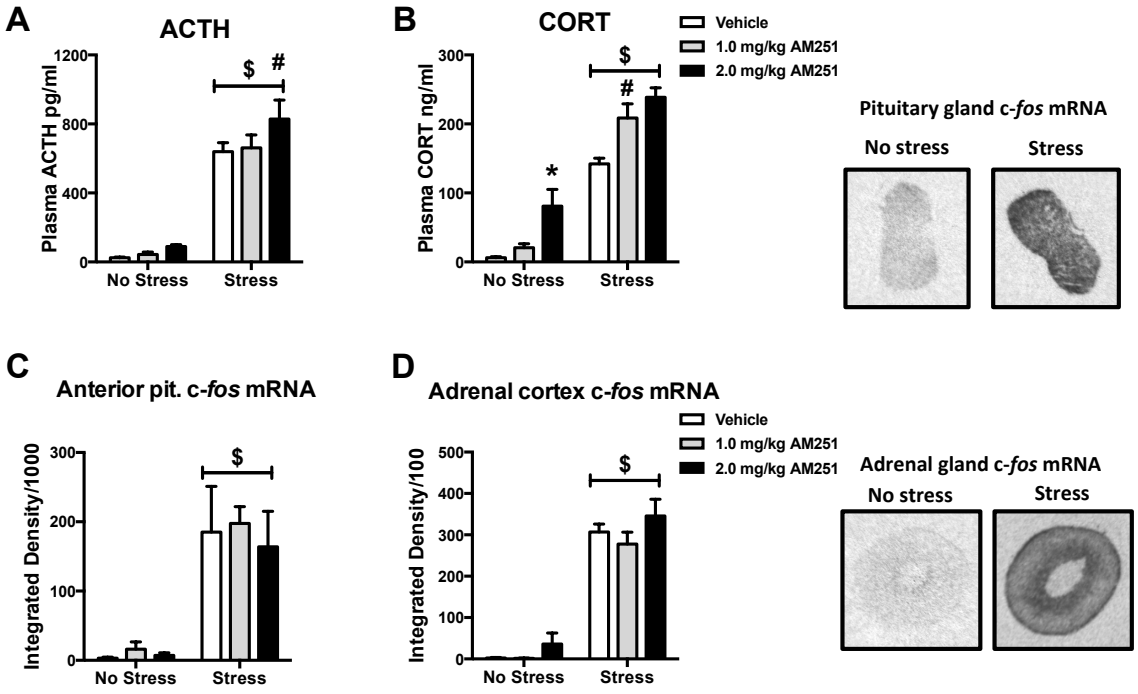

Figure 3.1. Peripheral HPA axis activity A. ACTH: Significant effect of stress ($, $p < 0.001$), Post hoc indication of potentiated response to stress in 2.0 mg/kg AM251-treated group (#, $p < 0.05$). **B.** CORT Significant effect of stress ($, $p < 0.001$) 2.0 mg/kg AM251 increased basal CORT level (*, $p < 0.05$) Post hoc indication of potentiated response to stress in 1.0 mg/kg AM251-treated group (#, $p < 0.05$). **C.** Stress increases c-*fos* mRNA in the anterior pituitary gland ($, $p < 0.001$) **D.** Stress increases c-*fos* mRNA in the adrenal cortex ($, $p < 0.001$) Representative autoradiographs of pituitary glands and adrenal glands demonstrate the differences between non-stressed and stress-induced levels of c-*fos* mRNA induction.

significant main effects of drug ($F_{(2,36)} = 19.37$, $p < 0.001$) and stress ($F_{(1,36)} = 203.7$, $p < 0.001$), but not a significant drug x stress interaction ($F_{(2,36)} = 1.79$, $p = 0.18$). Post hoc comparisons revealed that 2.0 mg/kg, but not 1.0 mg/kg AM251 treatment significantly elevated basal CORT compared to vehicle treatment ($p < 0.05$), indicative of disrupting tonic inhibition of the HPA axis at the level of the adrenal gland. Acute stress increased CORT in all three groups. Both doses of AM251 significantly elevated stress-induced

CORT compared to vehicle treatment. Significant elevation of stress-induced CORT by 1.0 mg/kg AM251 compared to vehicle treatment indicates a potentiation of stimulated HPA axis response. Immediate early gene c-*fos* mRNA was analyzed as an indicator of recent cellular activity in pituitary and adrenal glands. Analysis of anterior pituitary values with two-way ANOVA revealed a significant effect of stress ($F_{(1,38)} = 29.77$, $p < 0.001$), indicating that stress treatment increased c-*fos* mRNA in all treatment groups. There was no main effect of drug treatment ($F_{(2,38)} = 0.15$, $p = 0.86$), or interaction between drug and stress ($F_{(2,38)} = 0.07$, $p = 0.93$). Similarly, adrenal cortex analysis with two-way ANOVA revealed a significant increase in cellular activity from stress treatment ($F_{(1,38)} = 219.4$, $p < 0.001$) but not drug treatment ($F_{(2,38)} = 2.29$, $p = 0.11$), or interaction between the two ($F_{(2,38)} = 0.28$, $p = 0.76$).

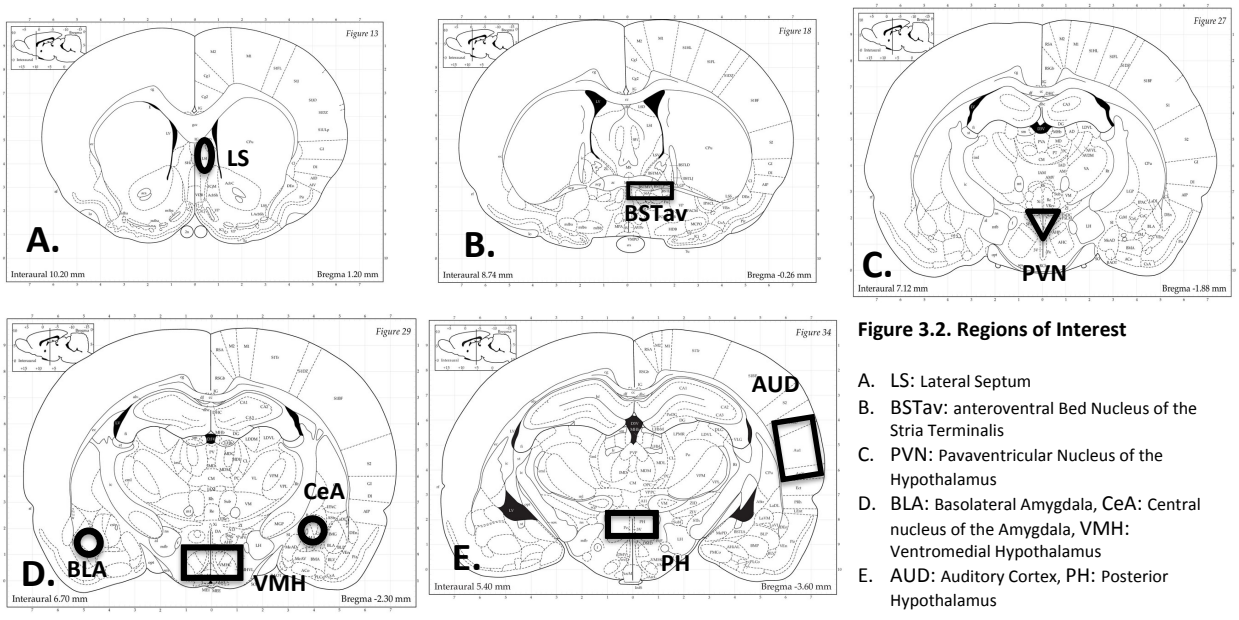

Figure 3.2. Regions of Interest

A. LS: Lateral Septum
B. BSTav: anteroventral Bed Nucleus of the Stria Terminalis
C. PVN: Pavaventricular Nucleus of the Hypothalamus
D. BLA: Basolateral Amygdala, CeA: Central nucleus of the Amygdala, VMH: Ventromedial Hypothalamus
E. AUD: Auditory Cortex, PH: Posterior Hypothalamus

Neural activity in stress-reactive sensory and limbic regions: Inhibitory tone and stress-reactivity

Stress-reactive sensory and limbic neural activity was also assessed by measure of c-*fos* mRNA expression in several brain regions (Figure 3.3). In the current study, remote administration of AM251 was found to increase c-*fos* mRNA in stressed and non-stressed rats, in dose-dependent and regionally variable patterns. Two-way ANOVA of PVN c-*fos* mRNA revealed significant effects of stress ($F_{(1,39)} = 118.6$, $p < 0.001$) and drug ($F_{(2,39)} = 7.75$, $p < 0.01$), but no significant stress x drug interaction ($F_{(2,39)} = 1.05$, $p = 0.36$) (Fig. 3.3A; For representative autoradiographs of PVN c-*fos* mRNA expression, see Fig. 3.4). Fisher's LSD post hoc comparisons of basal neural activity in the PVN between 2.0 mg/kg AM251 and vehicle treatment failed to reach significance ($p = 0.08$), but did indicate that this dose of AM251 resulted in higher c-*fos* mRNA overall compared to 1.0 mg/kg and vehicle. Post hoc comparisons confirmed that both doses of AM251 resulted in significantly higher neural responses to noise stress (1.0 mg/kg: $p < 0.05$, 2.0 mg/kg: $p < 0.001$). Two-way ANOVA of BLA c-*fos* mRNA values revealed significant effects of stress ($F_{(1,38)} = 8.20$, $p < 0.01$) and drug ($F_{(2,38)} = 8.80$, $p < 0.001$), but no significant stress x drug interaction ($F_{(2,38)} = 1.02$, $p = 0.37$). A similar dose-dependent pattern is visible in the BLA as in the PVN (Fig. 3.3B). Post hoc analyses detected a significant stimulation of neural activity from 2.0 ($p < 0.05$), but not 1.0 mg/kg AM251 ($p > 0.05$). Both doses resulted in significantly higher c-*fos* mRNA measure after loud noise stress when compared to vehicle controls (1.0 mg/kg: $p <$

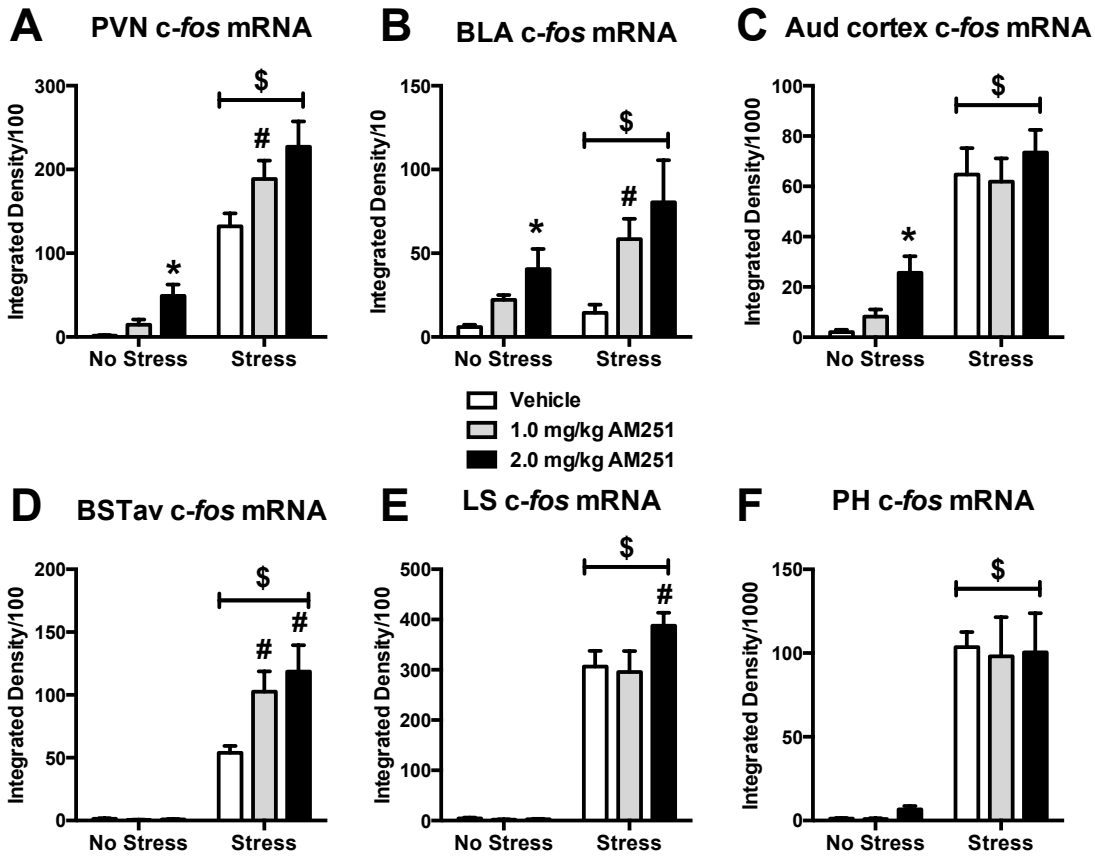

Figure 3.3. Neural activity in stress-reactive sensory and limbic regions: Inhibitory tone and stress reactivity. **A.** PVN: Paraventricular nucleus of the hypothalamus **B.** Basolateral amygdala **C.** Auditory cortex **D.** Anteroventral bed nucleus of the stria terminalis **E.** Lateral septum **F.** Posterior hypothalamus; Significant increase of immediate early gene c-*fos* mRNA by stress in all regions ($, $p < 0.01$); Increase in c-*fos* mRNA by AM251 in PVN, BLA and AUD in the absence of stress (*, $p < 0.05$) indicates disruption of CB1 receptor-mediated constitutive inhibitory tone. Potentiation of stress-induced c-*fos* mRNA by AM251 treatment indicates involvement of phasic eCB signaling in inhibitory regulation of psychoneuroendocrine stress reactivity (#, $p < 0.05$; indicated by significant interaction in the BSTav, and by post hoc comparisons in the PVN, BLA, and LS)

0.05, 2.0 mg/kg: $p < 0.001$). Stress treatment was not measured to stimulate c-*fos* mRNA in vehicle-treated rats ($p = 0.62$), but our results support an interpretation that antagonism of CB1 receptors facilitates stress-induced neural activity in this region. This

is visible in post hoc measures that indicate significant increase in c-*fos* mRNA in 1.0 mg/kg AM251-treated rats compared to vehicle in stressed rats, but not non-stressed rats. Though not necessary for our interpretation, a subsequent two way ANOVA with only two levels of drug treatment (vehicle and 1.0 mg/kg AM251) indicates significant drug x stress interaction from the lower dose of CB1 receptor antagonist ($F_{(1,26)} = 4.9$, $p < 0.05$).

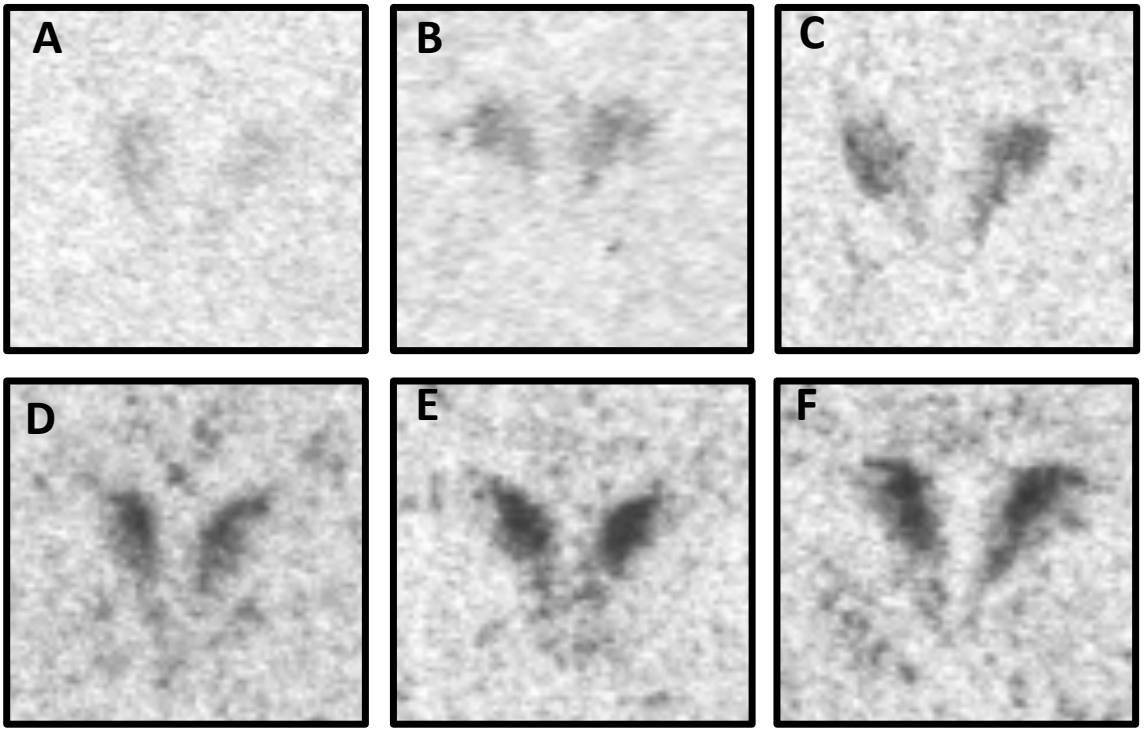

Figure 3.4 Paraventricular nucleus of the hypothalamus (PVN) c-*fos* mRNA. A. Vehicle-treated, non-stressed **B.** 1.0 mg/kg AM251-treated, non-stressed **C.** 2.0 mg/kg AM251-treated, non-stressed **D.** Vehicle-treated, acute loud noise stress **E.** 1.0 mg/kg AM251-treated, acute loud noise stress **F.** 2.0 mg/kg AM251-treated, acute loud noise stress. Acute noise stress significantly increases c-*fos* mRNA in the PVN. Post hoc comparisons indicate that 1.0 and 2.0 mg/kg AM251-treatments result in significantly higher stress-evoked induction than vehicle controls. In non-stressed rats, 2.0 mg/kg AM251 treatment results in a level of signal that is approximately 38% of that of vehicle-treated acutely stressed rats.

Auditory cortex c-*fos* mRNA was analyzed with two-way ANOVA, which indicated a significant effect of stress ($F_{(1,38)} = 77.6$, $p < 0.001$), and trend toward significant effect of drug ($F_{(2,38)} = 2.73$, $p = 0.078$). Post hoc comparisons found 2.0 mg/kg AM251 treatment to significantly increase c-*fos* mRNA compared to vehicle-treated controls ($p < 0.05$). PVN and BLA were found to display similar patterns to plasma CORT with CB1 receptor antagonism resulting in stimulation of basal and stress-induced activity. BSTav and LS results are presented in Figure 3.3, C and D. Two-way ANOVA revealed that in the BSTav, neither dose of remotely injected AM251 increased basal, non-stressed activity, but that both doses resulted in potentiation of stress-induced activity. Significant main effects of stress ($F_{(1,38)} = 91.5$, $p < 0.001$) and drug ($F_{(2,38)} = 4.09$, $p < 0.05$), and a significant stress x drug interaction ($F_{(2,38)} = 4.28$, $p < 0.05$) were obtained. A similar lack of stimulation of basal activity after remote CB1 receptor antagonist administration was measured in LS tissue. Two way ANOVA revealed significant effects of stress ($F_{(1,38)} = 284.2$, $p < 0.001$) but not drug ($F_{(2,38)} = 2.24$, $p = 0.12$), or significant stress x drug interaction ($F_{(2,38)} = 2.26$, $p = 0.12$). Post hoc analysis indicated a significant increase in stress-induced c-*fos* induction in 2.0 mg/kg AM251-treated rats compared to vehicle treated controls ($p < 0.05$), but no difference from this drug treatment in non-stressed rats. PH c-*fos* mRNA was significantly increased by stress ($F_{(1,36)} = 83.45$, $p < 0.001$), but was not found to be sensitive to AM251 treatment.

CB1 receptor mRNA: Multiple regions display alterations from stress or antagonist treatment

Type 1 cannabinoid receptor (CB1) mRNA alterations from acute stress have not been previously reported, but may have implications for repeated stress. We found bidirectional stress-induced alterations in CB1 mRNA in multiple limbic and HPA axis-intrinsic structures, as well as a more consistent pattern of alterations due to CB1 receptor antagonism alone (see Figure 3.5). Interestingly, we found bidirectional alterations in CB1 receptor mRNA due to antagonist administration and stress exposure to be dose-dependent in some regions, in a manner suggesting more subtle complexity to eCB system activity than previously reported. PVN CB1 receptor mRNA (Fig. 3.5A) was analyzed by two-way ANOVA, which revealed a significant interaction between stress and drug treatments ($F_{(2,38)} = 3.73$, $p < 0.05$), but no significant main effect of stress ($F_{(1,38)} = 0.01$, $p = 0.93$) or drug treatments ($F_{(2,38)} = 1.47$, $p = 0.25$). Post hoc comparisons indicate that acute loud noise stress significantly increases CB1 receptor mRNA. This stress-induced increase is prevented in both AM251 treatment groups, suggesting a possible need of eCB ligand binding at CB1 receptors for the stress-induced increase measured in vehicle controls. A stimulatory effect of the higher 2.0 mg/kg dose of AM251 on CB1 receptor mRNA in non-stressed rats is apparent, though post hoc measures failed to reach significance ($p = 0.13$). In the PVN, AM251 treatment appears to display opposite influences on CB1 receptor mRNA depending on dose, stress, and interaction of drug and stress. We found CB1 receptor mRNA in the anterior pituitary gland and cortex of the adrenal gland, though expression was light and unquantifiable in the anterior pituitary gland (data not shown). Adrenal cortex CB1 receptor mRNA displayed some patterns of stress and antagonist-related sensitivity that

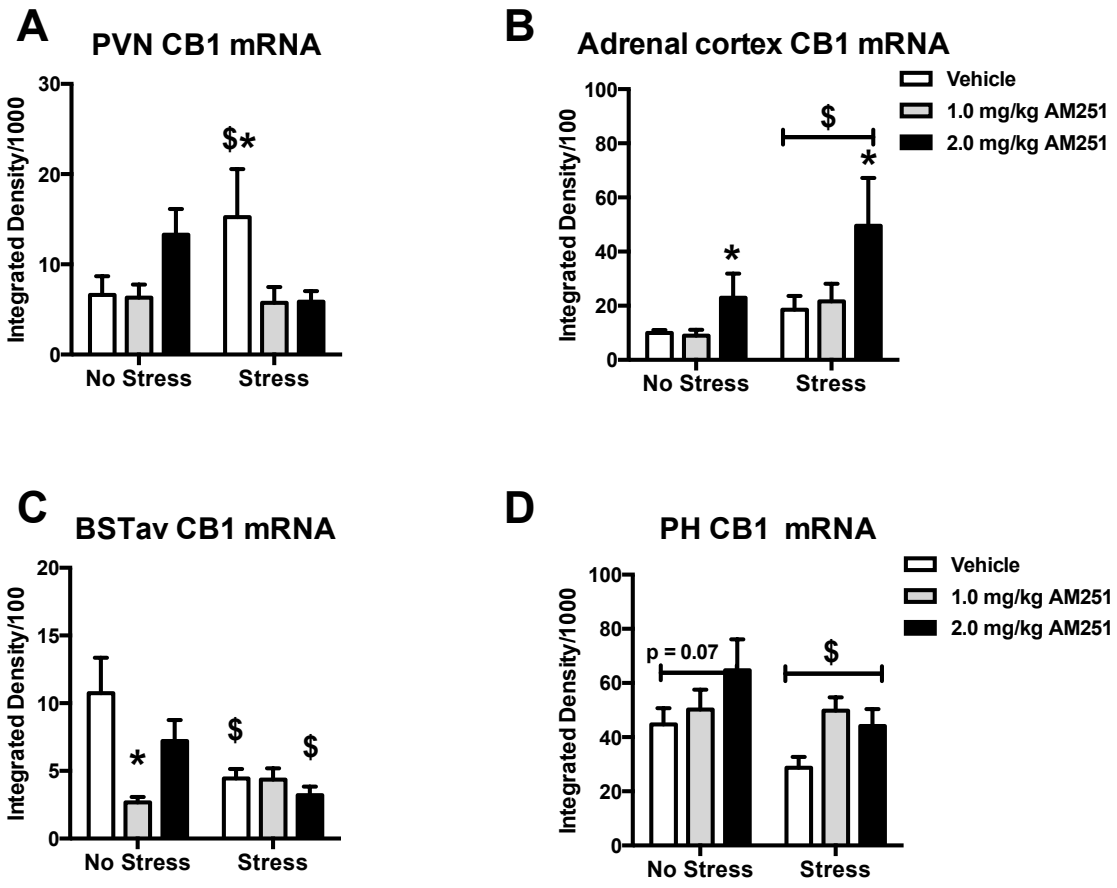

Figure 3.5 CB1 receptor mRNA is sensitive to acute stress and antagonist treatment in multiple regions. A. Paraventricular nucleus of the hypothalamus **B.** Cortex of the adrenal gland **C.** Anteroventral bed nucleus of the stria terminalis **D.** Anterior posterior hypothalamus. Acute loud noise stress increases CB1 receptor mRNA in the PVN and adrenal cortex ($, $p < 0.05$). Conversely, acute loud noise stress decreases CB1 receptor mRNA in the BSTav and PH ($, $p < 0.05$). A pattern of stimulation of basal CB1 receptor mRNA by 2.0 mg/kg AM251 treatment is apparent in all four regions, but does not always reach significance as analyzed (*, $p < 0.05$). An important contrast to this pattern is the lack of any evidence of this effect in rats treated with the lower dose (1.0 mg/kg) of AM251. 1.0 mg/kg AM251 seems to stabilize against increase and decrease in CB1 receptor mRNA levels whether in basal expression or in stress-induced alterations. In the BSTav, CB1 receptor mRNA expression is comparatively lower in the low dose, 1.0 mg/kg AM251-treated rats than in vehicle or 2.0 mg/kg AM251-treated rats (*, $p < 0.05$).

are similar to those measured in the PVN, with one clear distinction (Fig. 3.5B). Two-way ANOVA revealed significant main effects of stress ($F_{(1,39)} = 4.55$, $p < 0.05$) and drug

treatment ($F_{(2,39)}$ = 3.69, $p < 0.05$) but not stress x drug interaction ($F_{(2,39)}$ = 0.53, p = 0.59). The significant effect of stress indicates that acute loud noise stress increases CB1 receptor mRNA in all three treatment groups. This stress-induced increase was most pronounced in 2.0 mg/kg AM251-treated rats, which displayed higher levels compared to 1.0 mg/kg AM251 and vehicle-treated rats. A significant main effect of drug treatment indicated that 2.0 mg/kg AM251 increased CB1 receptor mRNA compared to the other treatment groups ($p < 0.05$). As in the PVN, the higher dose of AM251 led to an increase in CB1 receptor mRNA, but not the lower dose. Fisher's LSD multiple post hoc comparisons indicated significant increase in stress-induced CB1 receptor mRNA in

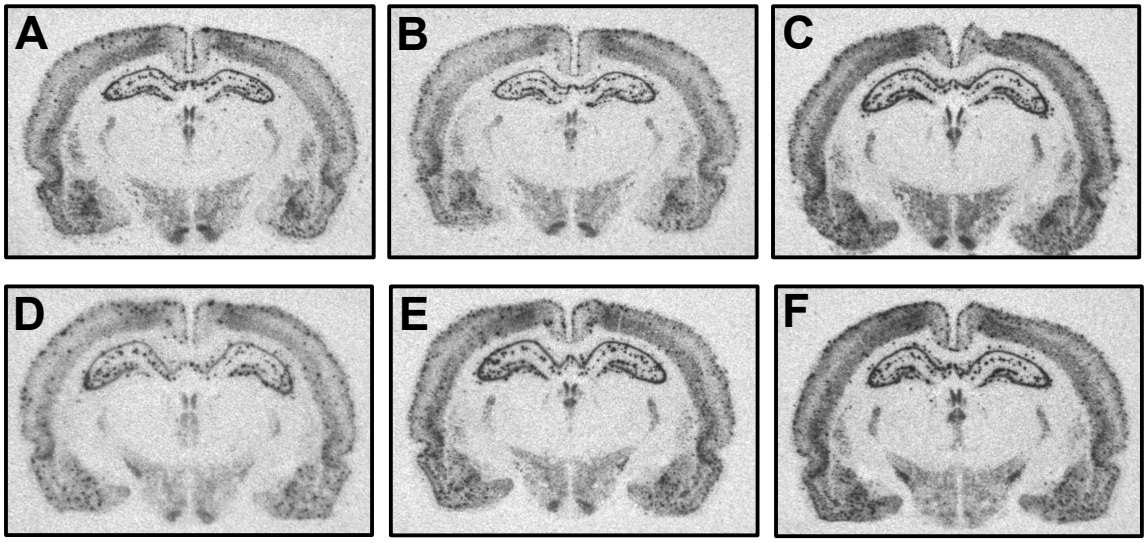

Figure 3.6 CB1 receptor mRNA in the anterior posterior hypothalamus A. Vehicle-treated, non-stressed **B.** 1.0 mg/kg AM251-treated, non-stressed **C.** 2.0 mg/kg AM251-treated, non-stressed **D.** Vehicle-treated, acute loud noise stress **E.** 1.0 mg/kg AM251-treated, acute loud noise stress **F.** 2.0 mg/kg AM251-treated, acute loud noise stress. Acute loud noise stress decreases CB1 receptor mRNA in the anterior PH.

rats administered the higher dose of AM251 compared to the other two treatment groups ($p < 0.05$), but did not detect an increase from this dose in non-stressed rats. Adrenal cortex CB1 receptor mRNA values indicate that this measure increases with adrenal gland activity in a pattern similar to that measured in plasma CORT. Stress and antagonist treatment both independently result in increased CB1 receptor mRNA expression, and these influences can combine to potentiate the stimulation. Interestingly, inhibition of CB1 receptor mRNA increase by AM251 was not visible in the adrenal cortex as it was in the PVN. Analysis of BSTav CB1 receptor mRNA revealed significant main effects of stress ($F_{(1,41)} = 6.62$, $p < 0.05$) and drug ($F_{(2,41)} = 4.63$, $p < 0.05$), and a significant stress x drug interaction ($F_{(2,41)} = 4.67$, $p < 0.05$). In vehicle treated rats, stress significantly decreased CB1 receptor mRNA ($p < 0.01$). This stress-induced decrease was also measured in rats receiving the higher dose (2.0 mg/kg) of AM251 ($p < 0.05$). The lower dose (1.0 mg/kg) of AM251 significantly decreased basal CB1 receptor mRNA compared to vehicle and 2.0 mg/kg AM251 ($p < 0.05$), and this level of expression was not changed by stress treatment. PH CB1 receptor mRNA was analyzed with two-way ANOVA, which revealed a significant effect of stress ($F_{(1,35)} = 4.25$, $p < 0.05$) and a trend toward significant effect of drug ($F_{(2,35)} = 3.61$, $p = 0.054$) but not a significant interaction. Stress decreased CB1 receptor mRNA in the PH, and 2.0 mg/kg AM251 treatment in non-stressed rats was found to trend toward increasing CB1 receptor mRNA (Fig. 3.5D, 3.6A-F). The stress-induced decrease in CB1 receptor mRNA measured in the BSTav contrasts with the stress-induced increases measured in the PVN and adrenal cortex. CB1 receptor mRNA expression in the BLA, CeA , LSd,

and VMH was not significantly altered by stress or drug treatments ($p > 0.05$ for each, Fig. 3.7A-D).

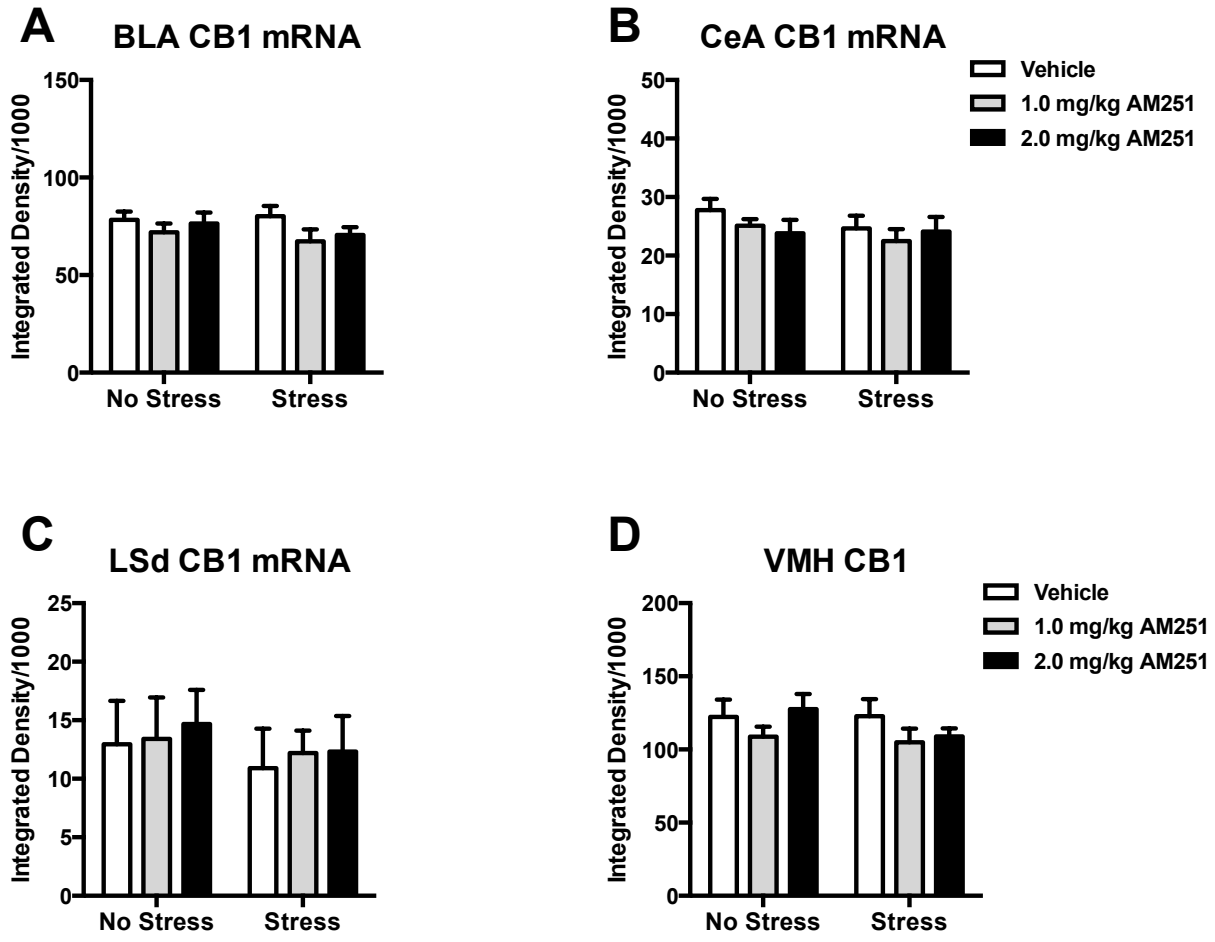

Figure 3.7 CB1 receptor mRNA was not sensitive to stress or antagonist treatment in other limbic regions. BLA: basolateral amygdala, CeA: central amygdala, LSd: dorsal lateral septum, VMH: ventromedial hypothalamus

Discussion

The results of our study provide novel information and perspective that contribute to the understanding of a role of the eCB system in psychoneuroendocrine regulation.

We used remote administration of AM251 through i.p. catheters to ensure that effects produced were not confounded by the stress of injection-related pain or experimenter handling. Generally, data from this study confirm the results and conclusions of our previous report, and indicate that the stress of injection was not a major causative factor in the increases in neural and endocrine activity measured after i.p. injection of 2.0 mg/kg AM251. The increases in c-*fos* mRNA expression and plasma CORT measured after CB1 receptor antagonism with AM251 provide strong evidence for the presence and regional specificity of a constitutive CB1 receptor-dependent inhibition of basal activity of the HPA axis and in multiple neural regions. Though the dose-dependency of some CB1 receptor antagonists to directly stimulate neuroendocrine activity has been acknowledged and strategically utilized in previous eCB system research, our study is the first to include direct comparison of two systemic doses of AM251 in comprehensive examination of basal and acute stress-induced limbic and HPA axis activity. This comparison provides an important groundwork for future studies and a functional frame of reference for understanding the relative contributions and interactions of CB1 receptor activity in individual neural regions and levels of the HPA axis. Results of our study also contribute to improving the understanding of the actions of AM251 as a research tool for examining the eCB system. The distinct lack of AM251-induced neural activity in the LS and BSTav contrasts with the confirmed ability of the CB1 receptor antagonist to stimulate activity in the BLA, AUD, and PVN and indicates that inverse-agonist like effects of AM251 may be more dependent on important regional variation in eCB system activity than an inherent property of AM251. Also, we report for the first

time that CB1 receptor mRNA is quickly sensitive to both CB1 receptor antagonism and acute psychological stress in adrenal tissue and neural regions involved in acute stress-reactivity. We measured an unexpected bidirectional alteration of CB1 receptor mRNA expression in patterns that were dose-dependent and regionally variable. Overall, the results of our study contribute to the growing understanding that eCB activity contributes to multiple aspects of neural and HPA axis regulation, and supports a need for specific attention to dosage when designing and interpreting research on the eCB system.

Role of the eCB system in psychoneuroendocrine reactivity to acute stress

Results of this study are in agreement with the currently well-accepted role of the eCB system as a multifaceted inhibitory buffer of stress-induced neural and HPA axis reactivity to psychological stress. Our data support a view that this inhibitory mechanism is dependent on integrative contribution of CB1 receptor activity in multiple regions in the brain and body. As in our previous report, disruption of CB1 receptor activity with AM251 treatment before acute loud noise stress exposure resulted in potentiation of stress-reactive responses in multiple limbic regions in the brain and in measures of HPA axis response. The BSTav displayed the strongest pattern of potentiated stress reactivity, with both doses of AM251 resulting in increased c-*fos* mRNA compared to vehicle treatments. The LS, BLA, and PVN similarly displayed patterns of potentiated stress-induced neural activity resulting from CB1 receptor antagonism. The LS appears to be less sensitive to potentiation by CB1 receptor antagonism than the BSTav, as the lower dose of AM251 (1.0 mg/kg) did not result in increased stress-induced neural

activity beyond vehicle-treated controls, compared to robust potentiation by this dose in the BSTav. Evidence for stress-induced potentiation of LS activity was only detected at the higher dose of AM251 (2.0 mg/kg) in the current study. Also, in our previous study, the LS analysis indicated a trend toward potentiation of stress-induced activity after 2.0 mg/kg AM251 treatment, though in Sachin Patel's earlier Fos mapping study in 2005, this region displayed strong SR141716A-evoked potentiation of restraint stress-induced Fos protein in mice (Newsom et al., 2012; Patel, Roelke, Rademacher, & Hillard, 2005). The BSTAV and LS can independently influence psychoneuroendocrine stress-reactivity (Choi et al., 2008; 2007; Reis, Scopinho, Guimarães, Corrêa, & Resstel, 2011) and may mediate between the amaygdala and hypothalamus in basal and acute stress-induced neuroactivity (Crestani et al., 2013; Herman, 2013; Hill & Tasker, 2012; Hill, McLaughlin, et al., 2010a; Patel & Hillard, 2008; M. S. Weinberg, Johnson, Bhatt, & Spencer, 2010). We previously reported these structures to be distinctly absent of c-*fos* mRNA induction after IP injection of 2.0 mg/kg AM251 to non-stressed rats, and find c-*fos* mRNA induction in both to correlate with stressor intensity and HPA axis responses to acute noise stress (Burow et al., 2005; Newsom et al., 2012). The absence of antagonist-induced c-*fos* mRNA in these regions and robust stress-reactivity makes them valuable measures of general central stress-reactivity in stress research. Independent contribution of eCB signaling in these regions to psychoneuroendocrine stress-reactivity will require further examination, but the results of this study support their involvement in a collective multi-structural contribution to neuroendocrine regulation.

The PVN, and BLA are two stress-reactive neural regions that we previously measured to have robust c-*fos* mRNA induction after i.p. injections of 2.0 mg/kg AM251 (Newsom et al., 2012). c-*fos* mRNA in the PVN was previously shown to positively correlate with acute stressor intensity, as well as HPA axis hormone responses (ACTH, CORT) (Burow et al., 2005). The BLA has been the focus of eCB stress-reactivity research (Bedse et al., 2014; Gray et al., 2015; Ramikie & Patel, 2012). We do not always find this region to display strong stress-induced c-*fos* mRNA (Burow et al., 2005; Campeau et al., 2002; Newsom et al., 2012; Sasse, Nyhuis, Masini, Day, & Campeau, 2013; M. S. Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008b), but measured robust increase after i.p. injections of 2.0 mg/kg AM251 (Newsom et al., 2012), supporting a presence of constitutive inhibitory eCB tone in this region (Hill et al., 2009b). The inclusion of an additional, lower dose of AM251 in this study revealed evidence of potentiated stress reactivity in the BLA and PVN. This potentiation is not detectable after administration of 2.0 mg/kg AM251, which results in significant induction of c-*fos* mRNA in the absence of stress that likely occludes the evidence of potentiation of stress-induced activity, and indicates that tonic and phasic eCB-dependent actions are likely regulated by distinct mechanisms (Di et al., 2013; Hill et al., 2009b). It is also possible that direct stimulation of activity in the BLA and PVN by AM251 could be misidentified as activity relating to acute behavior or experience, which suggests a need for careful selection of dose and inclusion of control groups in research involving local microinjection of CB1 receptor antagonists into these regions. Examination of local CB1 receptor-dependent neuroendocrine regulation in the PVN has led to mixed results.

Electrophysiology studies support an inhibitory role of CB1 receptors in this region (Di, Malcher-Lopes, Halmos, & Tasker, 2003; Wamsteeker et al., 2010), but in vivo studies have proven this to be difficult to detect in HPA axis reactivity to stress (Evanson & Herman, 2015; Evanson, Tasker, Hill, Hillard, & Herman, 2010).

In contrast to our previous experiment, we did not measure strong stress-induced potentiation of plasma ACTH or c-*fos* mRNA in the anterior pituitary gland in the current experiment, though post hoc comparisons indicated a potentiation of ACTH resulting from 2.0 mg/kg AM251. As measures of HPA axis stimulation in response to acute psychological stress typically correlate positively with stress-induced activity of neural regions such as the LS, BST, and PVN (Burow et al., 2005), it is likely that these measures do not accurately reflect the potentiation of HPA axis response that would be expected based on the pattern of activity measured in multiple neural regions. However, plasma CORT and adrenal cortex c-*fos* mRNA analysis in the current study did provide evidence of AM251-dependent potentiation of stress reactivity that is detectable after administration of a lower dose. These data support conclusions of previous research (D. P. Finn, 2010; Hill & Tasker, 2012; Lutz, 2009; Ramikie & Patel, 2012; Riebe & Wotjak, 2011; Sorrells & Sapolsky, 2007b) and the interpretations that the ability of CB1 receptor blockade to potentiate stress-induced activity supports a role of activity-dependent stimulation of eCB activity at CB1 receptors in dampening psychoneuroendocrine reactivity to acute stress. Further, our results indicate that disruption of eCB signaling at CB1 receptors by chronic stressful experience or glucocorticoid exposure (Bowles et al., 2012; Campos et al., 2013; Hill, Carrier, Ho, et

al., 2008a; Wamsteeker et al., 2010; Xing et al., 2014) would lead to hypersensitivity to stress and may be a causative factor in stress-related pathology (Hill & Patel, 2013; Hillard et al., 2012). The potential contribution of our unique study design on HPA axis measures in this study is discussed in detail in the following section.

eCB-mediated tonic inhibition of neural and endocrine activity

The specific mechanisms of tonic inhibition by constitutive eCB system activity are not well understood. A small amount of evidence supports presence of inhibitory tone in the BLA (Hill et al., 2009b; Katona et al., 2001; Newsom et al., 2012; Trezza et al., 2012), multiple regions of the cortex (Newsom et al., 2012; Singh, Verty, Price, McGregor, & Mallet, 2004), and in the HPA axis (Cota, 2007; Hill et al., 2009b; Newsom et al., 2012; Patel, 2004c). The results of the current study confirm this mechanism in the BLA and AUD, and indicate that the activity we previously reported to result from CB1 receptor antagonism was not dependent on the stress of i.p. injection procedures. PVN data in this study do not appear to reflect as strong an influence of local CB1 receptor-mediated inhibitory tone as we previously measured (Newsom et al., 2012). As stress-independent PVN activity resulting from CB1 receptor antagonism is a main topic of interest in this study, this interpretation requires careful consideration. Our ultimate interpretation is that the results of this study support the presence of eCB-mediated inhibitory tone at the PVN.

A possible reason for this difference is a minor alteration in quantification strategy in this study compared to our prior study. We measured and reported c-*fos* mRNA

signal in our previous study, based on a method of directly tracing the visible signal in the autoradiograms used for analysis. The current study utilized a single representative template for quantification of each section used in developing a mean score for each individual rat, which was then included in a group average. The use of a template allows inclusion of a measure of the area, or size of the PVN representation in the value for each section, which is then multiplied by the signal value to provide an integrated density measure (presented in arbitrary units). The integrated density numbers from 6-8 autoradiograms, which each represent individual tissue sections collected from this region, are averaged together for a single representative value for each rat. For PVN (a relatively small region) analysis, I have started to use only the top 4-6 integrative values for each rat to calculate a mean value for the final representative score, as a way to ensure that all values are taken from the largest, most representative region of the PVN. This method provides the most representative densitometric values and overall analysis, and represents an improvement from the strategy utilized in our previous report.

However, additional factors should be considered in drawing conclusion on CB1 receptor-mediated tonic inhibition from the data patterns in each of our two studies. The quantification strategy used in our previous study likely over-represented the c-*fos* mRNA induction resulting from AM251 treatment in non-stressed rats in comparison to that of the induction representative of vehicle treatment and acute stress, as it removed the influence of size of the representative PVN signal in the analysis. We've since realized that size of the representative autoradiographic signal is importantly indicative of signal induction, if all tissue is collected from the ideal region of interest. The ability of

2.0 mg/kg AM251 to result in c-*fos* mRNA induction greater than that observed to result from moderate intensity loud noise stress (95 dBA), is an overestimation. In the current study, the same dose of AM251 was found to result in a level of PVN c-*fos* mRNA induction in non-stressed rats that was measured to be approximately 38% of the stress-induced value in vehicle-treated controls. This value is not found by two-way ANOVA and multiple post-hoc comparisons to be distinctly different from the level of c-*fos* mRNA induction measured in non-stressed, vehicle control rats ($p = 0.08$). Though, this difference is somewhat supported by post hoc analysis of a main effect of drug, which found 2.0 mg/kg AM251 treatment to result in higher c-*fos* mRNA values compared the other treatments when stress treatment is not considered (no difference was detected between 2.0 and 1.0 mg/kg treatments in stress-induced expression). The sensitivity of our statistical analysis is brought into question by this issue. We used two-way ANOVA along with the relatively liberal Fisher's Least Significant Difference (LSD) test for all post hoc comparisons in this study, but are lacking in the descriptive capability that would benefit this study. The PVN c-*fos* induction resulting from 2.0 mg/kg AM251, being approximately 38% of the level measured in the acute stress vehicle-treated control group, is much greater than the level observed in the non-stressed, vehicle-treated control group, which displayed 1% of the induction measured in the acute stress, vehicle treatment group. We outline this issue to justify a conclusion that 2.0 mg/kg AM251 treatment did, in fact increase PVN c-*fos* mRNA induction, and to highlight an inherent issue of reduced descriptive sensitivity in research study designs such as that used in the current study, which are contrived to examine dose-dependent

effects and include all appropriate control groups. Still, the amount of PVN activity induced by 2.0 mg/kg AM251 is less than that stimulated by acute stress, and lower than we expected given the results of our initial study. An additional possibility is that HPA axis measures in this study were affected by the additional complications in study design, which included i.p. catheter implantation surgery, and required overnight housing of the rats in the loud noise boxes, which necessitated connection to the catheter hardware. Both of these additional manipulations are stressors that would activate the HPA axis, and may have contributed to a reduction in sensitivity of the related measures on the testing day due to inducing negative feedback mechanisms (Dallman et al., 1987; Martí & Armario, 1998; McEwen, 1998).

Peripheral CB1 receptor-dependent neuroendocrine regulation

Consistent with our previous report, 2.0 mg/kg AM251 significantly elevated plasma CORT in the absence of stress, and we can rule out the influence of the acute stress of injection on this measure. This was detected despite a lack of significant elevation of anterior pituitary gland activity (ACTH, c-*fos* mRNA) by the same treatment, and supports a presence of tonic inhibition of activity at the level of the adrenal gland that is dependent on CB1 receptors. Plasma CORT was potentiated after acute loud noise stress at the lower 1.0 mg/kg dose, which did not significantly elevate plasma CORT in the absence of stress. This supports a contribution of adrenal level CB1 receptor activity in neuroendocrine reactivity to stress. Together, these results support a role of CB1 receptor inhibition in tonic and phasic neuroendocrine regulation. The

anterior pituitary gland was not stimulated by AM251 in non-stressed rats, as indicated by measures of ACTH and c-*fos* mRNA, but did not reflect the strong potentiation of activity we measured in our previous study. This lack of activity suggests that the PVN c-*fos* mRNA induction by AM251 is not related to acute HPA axis activity. Shi Di and colleagues have recently published a study on eCB-mediated inhibitory tone in the hypothalamus, with a thoughtful review of their related data in the discussion section (Di et al., 2013), which indicates that CB1 receptor-dependent tonic inhibition in the PVN is predominantly regulating magnocellular neurons. A strong possibility is that the AM251-mediated c-*fos* mRNA induction in the PVN likely reflects disinhibition of magnocellular neurons, rather than CRH-releasing parvocellular neurons, though this measure could be indicating c-*fos* mRNA induction in parvocellular neurons that is unrelated to corticotropin-releasing hormone (CRH) release.

CB1 receptor mRNA expression and dynamic sensitivity to AM251 and acute stress

CB1 receptor mRNA was found to be sensitive to both antagonism by AM251 and acute stress, in various tissues. The BSTav was measured to exhibit stress-induced decrease in CB1 mRNA that was visible in vehicle treatment groups and in 2.0 mg/kg AM251 treatment groups, as well as a decrease from 1.0 mg/kg AM251 in non-stressed rats compared to vehicle controls and 2.0 mg/kg AM251. Interestingly, the decrease in CB1 receptor mRNA from low dose CB1 receptor antagonism contrasts with the lack of antagonist-specific induction of c-*fos* mRNA. The relative increase in CB1 receptor

mRNA resulting from the higher 2.0 mg/kg AM251 treatment in non-stressed rats matches the pattern observed in both the PVN and adrenal cortex, which gives weight to the validity of this measure. However, the PVN and adrenal cortex both reflected stress-induced increase in CB1 receptor mRNA, which is the opposite of what was measured in the BSTav. An increase in functional CB1 receptors in a tissue would likely increase sensitivity to inhibitory eCBs, therefore decreasing excitability of this region. It makes sense that HPA axis intrinsic structures would display this pattern, as negative feedback is a strong theme (Pace, Gaylord, Jarvis, Girotti, & Spencer, 2009; Sapolsky, 1996) in this axis. As chronic stress has been demonstrated to impair negative feedback of the HPA axis (Checkley, 1996; Holsboer, 2001; McEwen, 2004), as well as disrupt eCB system functioning in the PVN (Wamsteeker et al., 2010), it appears likely that disruption of CB1 receptor functioning is a contributing factor to HPA axis dysregulation resulting from chronic stress. The stress-induced decrease in CB1 receptor mRNA measured in the BSTav would potentially result in an increased state of neural excitability if this mRNA pattern indicates an eventual decrease in functional CB1 receptors in this region. It will take more research to determine what these alterations mean, and if there is a different time period after stress or CB1 receptor antagonism that will display additional alterations, or a more uniform pattern. Overall, the results of this study support a multi-faceted role of the endocannabinoid system in psychoneuroendocrine regulation involving both central and peripheral CB1 receptors and including inhibitory contribution to tonic and phasic actions. The CB1 receptor mRNA alterations by stress and antagonism may indicate an involvement of this system

in alterations relating to repeated stress. Potential difficulties in statistical analysis arising from a large number of treatment groups makes dose-response comparisons difficult to include in every study, which highlights the importance of careful mapping studies. Additionally, the stress of injection is not an obvious contributor in eCB system psychoneuroendocrine regulation and is not a necessary design for future studies.

Chapter 4

Endocannabinoid signaling as an intrinsic component of the circuits mediating adaptive responses to repeated stress exposures

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Abstract

Evidence implicates the endocannabinoid (eCB) system as a negative modulator of neural and endocrine responses to acute stressors. Recently, eCBs were also shown to influence the development of habituated hypothalamo-pituitary-adrenal (HPA) axis responses to repeated homotypic stress, in which repeated exposures to the same stressor reliably decreases subsequent activation of the HPA axis. The present studies were initiated to distinguish a potential role of eCB signaling in the acquisition as compared to the expression of habituated HPA axis responses. Adult male Sprague Dawley rats (Harlan) were exposed to daily, 30-minute sessions of loud white noise (95 dB) for 8 days. Rats received either the cannabinoid receptor 1 (CB1) antagonist AM251 (2mg/kg or 0.5mg/kg, i.p.) or vehicle 30-min before the loud noise sessions on the first 7 days, but not on the 8th day of stress exposure. Blood samples were taken after loud noise on days 1, 4, 7, and 8. The rats treated with 2 mg/kg AM251 had higher levels of plasma corticosterone (CORT) on days 1,4, and 7, and a slower rate of habituation than rats receiving vehicle or 0.5 mg/kg injections of AM251. However, on day 8 of noise (drug-free), 2 mg/kg AM251-treated rats had near complete attenuation of HPA axis response, as observed in rats receiving vehicle or 0.5 mg/kg AM251 injections during the initial 7 loud noise exposures. This suggests that disruption of CB1 receptor signaling does not disrupt the plasticity associated with acquisition of habituation to repeated homotypic stress. In a second study, rats were exposed to daily loud noise stress for 7 days to establish HPA axis habituation without drug treatments. Twenty-four hours later, rats were injected with 1 mg/kg AM251 or vehicle, 30 min prior to a final 30-

min loud noise exposure. Vehicle-treated rats displayed reliable habituation of HPA axis response, but CB1 antagonism disrupted the expression of this habituated response, by restoring plasma CORT to levels observed during the initial (day 1) loud noise exposure. Rats were given an additional mild stress exposure (novel environment) 24-hours later, along with a cohort of rats not previously habituated to noise stress. 1 mg/kg AM251 treatment prior to novel environment resulted in a significantly larger HPA axis response, but only in rats recently habituated to repeated loud noises. A third study was performed with similar design to the second study with a few minor alterations. We added a treatment group with a lower dose of AM251 (0.5 mg/kg) to ensure that the effects of novel stressor sensitization and disruption of the expression of habituation were independent of the potential confound of acute stimulation of stress-reactivity. Rats were exposed to 7 sessions of daily loud noise stress to establish habituation, and on day 8, the ability of 0.5 and 1.0 mg/kg AM251 to reveal novel stressor sensitization was tested with acute restraint stress. The same treatments were administered before a final loud noise exposure on day 9, and rats were sacrificed for collection of trunk blood and brains. Both doses of AM251 resulted in potentiated responses to restraint stress in rats that had repeated noise exposure, and significantly disrupted the expression of HPA axis habituation on day 9. Analysis of c-*fos* mRNA induction indicated that 0.5 mg/kg AM251 did not potentiate neural reactivity to the to loud noise stress if administered before the initial exposure, but did increase c-*fos* mRNA responses in several limbic regions when administered to rats before the 8th loud noise stress exposure. These results point to eCB signaling as an intrinsic component of the circuits expressing

adaptive responses to repeated stress, without itself mediating the plasticity associated with repeated stress exposures.

Introduction

Habituation of responses to repeatedly experienced psychological stressors is an adaptive mechanism that can limit the accumulation of stress-related pathology (Herman, 2013). Impaired ability to habituate to stressors has been reported in populations with mood and anxiety disorders such as depression, anxiety, and post-traumatic stress disorder (Brierley & Jamieson, 1974; Chattopadhyay et al., 1980; LADER & WING, 1964; Thomson & Craighead, 2008), and this impairment may contribute to initiation and maintenance of these disorders. Treatment strategies for stress-related disorders related to correction or improvement of the ability to appropriately habituate to repeatedly experienced stressors may be beneficial, but little is currently known about the neural circuitry and mechanisms responsible for this type of plasticity. Stress habituation has been reported to simultaneously lead to sensitized responses to novel stressors, though it is unclear if these response patterns are a result of habituation-related plasticity (Grissom et al., 2008; M. S. Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008b). Further examination of the neural circuitry involved in habituation and sensitization of reactivity to psychological stress is necessary to develop strategies for reducing stress-related damage.

The endogenous cannabinoid (eCB) system negatively modulates neural and hormonal responses to acute psychological stress (Hill & McEwen, 2010; Newsom et al., 2012; Patel, 2004a; Riebe & Wotjak, 2011) and may be protective against repeated stress-related pathology (Hill & Patel, 2013). Indeed, increases in endocannabinoid signaling at CB1 receptors contribute to the habituation of neural and hormonal

reactions to repeatedly experienced psychological stressors (Hill, McLaughlin, et al., 2010a; Patel et al., 2005; Patel & Hillard, 2008), but additional evidence, however suggest that multiple neural regions and mechanisms influence stress habituation (Herman, 2013) likely increasing the complexity of the underlying circuitry. Using rodent models, Hill et al., (Hill, McLaughlin, et al., 2010a), and Patel et al., (Patel et al., 2005) both demonstrated CB1 receptor antagonism to disrupt the habituated responses after repeated restraint stress, and also measured increases in eCB 2-arachidonoyl glycerol (2AG) in the basolateral amygdala (BLA) after repeated stress which could mediate the habituation of the hypothalamic-pituitary-adrenal (HPA) axis responses characteristic of habituation to psychological stressors. Additionally, CB1 receptor knockout (KO) models have been demonstrated to display disrupted habituation to stressors (Fride, Suris, Weidenfeld, & Mechoulam, 2005; Kamprath et al., 2006), but it is difficult to separate the contribution of acute deficiency of eCB signaling or developmental effects from habituation-specific involvement of the eCB system in receptor KO models.

Disruption of neural eCB signaling resulting from chronic stress could contribute to the sensitization of neuropsychological and hormonal responses to later stressful experience (Hill & Patel, 2013). Antagonism of CB1 receptors as well as CB1 receptor KO both facilitate neural, behavioral, and hormonal responses to acute stress, and limbic eCB signaling is disrupted in some models of repeated stress as well as chronic glucocorticoid exposure (Hill & Patel, 2013). It is unclear if this mechanism of sensitization is related to the cross-sensitization of stress-reactivity that co-occurs during some repeated stressor paradigms and how this relates to the plasticity

responsible for habituation to repeated homotypic stress (Bhatnagar & Dallman, 1998; Grissom et al., 2008).

We designed the current experiments to distinguish between the involvement of the eCB system in the plasticity necessary for acquisition of habituation to repeated stress from involvement specifically in the performance of the characteristic reductions in neuroendocrine responses (Campeau et al., 2002; 2008). In addition, the potential involvement of the eCB system was examined in the sensitization of neuroendocrine stress-reactivity that can co-occur during development of homotypic stressor habituation.

Materials and Methods

Subjects:

One hundred and sixteen male Sprague-Dawley rats (Harlan, Indianapolis IN) weighing 275–300 grams upon arrival were used. Animals were housed in polycarbonate tubs containing wood shavings, with wire lids providing rat chow and water ad libitum. Conditions in the animal colony were controlled to constant humidity and temperature, with a 12:12 hour light/dark cycle (lights on at 7:00 am). Testing was performed between 8:30 am and 12:30 pm during the circadian nadir for the HPA axis. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and conformed to the United States of America National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

Experimental Design:

Experiment 1: *Daily pre-injection of CB1 receptor antagonist AM251 before loud noise stress to examine role of eCB signaling in acquisition of the necessary habituation plasticity compared to the performance of habituation.*

Rats were randomly assigned to one of three groups (n = 8/group) to receive intraperitoneal (i.p.) injection of CB1 receptor antagonist AM251 at high dose (2 mg/kg), low dose (0.5 mg/kg), or vehicle thirty minutes before each of seven days of 30 minutes of loud noise stress exposure (95 dBA). This regimen of loud noise stress exposures consistently induce total or near total habituation of HPA axis responses (Masini, Day, & Campeau, 2008). Blood samples were collected immediately after stress exposures on Day 1, 3, 7, and 8, using heparinized capillary tubes and a small tail nick at the base of the tail. On the 8th day of loud noise exposure, rats were not given drug pre-treatment to test the acquisition of habituation. Plasma was used to measure CORT levels.

Experiment 2: *Use of low dose of CB1 receptor antagonist AM251 pretreatment before 8th day of loud noise exposure and before heterotypic stressor exposure to assess CB1 receptor modulation of the expression of habituated responses and response sensitization to a mild, heterotypic stressor.*

Rats (n = 18) were exposed to daily, 30-minute loud noise stress sessions (95 dBA) for seven days to stabilize habituated HPA axis responses. An additional control group of rats (n = 3) were transported from the colony daily, and placed into quiet noise chambers for the same amount of time before serving as an acute stress treatment group on day 9. Blood samples were taken via tail nick after the first day of loud noise stress for determination of acute CORT release. On day 8, rats were randomly assigned to one of two groups (n = 9/group) to receive pre-injection of CB1 receptor antagonist AM251 at a low dose (1.0 mg/kg) reported to be without acute facilitation of HPA axis response to restraint stress (Hill, McLaughlin, et al., 2010a), or vehicle before a final loud noise stress exposure. Blood samples were taken immediately following stress treatment. On day 9, rats were again given pretreatment of low dose AM251 (1.0 mg/kg) or vehicle 30 minutes before being exposed to a mild heterotypic psychological stressor (15 minutes in a novel environment) to examine a possible sensitization of stress-reactivity resulting from the previous 8 days of loud noise stress. To ensure absence of a confounding additive effect of AM251 treatment from day 8 to day 9, group assignments (vehicle/AM251) for roughly half of the repeated stressed rats were arbitrarily switched to the other treatment before day 9 testing. Control rats without the recent repeated stress history were pre-treated with the low dose of AM251 before acute loud noise stress. Blood samples were taken from all rats immediately following loud noise stress exposure on day 9. Plasma CORT was later measured as an index of stress-reactivity. Based on the results of Experiment 1, our hypothesis was that CB1 receptor antagonism (1 mg/kg AM251) would disrupt the expression of habituated HPA

axis response on day 8 of loud noise stress. Detection of an AM251-induced sensitization of HPA axis response to novel environment stress in repeatedly stressed rats, but not in rats without repeated loud noise stress experience, was expected to offer evidence for sub-threshold acute potentiating ability of this specific AM251 dose on psychological stress reactivity.

Experiment 3: *Examination of generalizability of repeated stress-related cross-sensitization to restraint stress, and comparison of stress-dependent neural activity of CB1 receptor antagonism in acute and habituated stress.*

Pilot testing for this experiment included an additional lower dose of CB1 receptor antagonist AM251 (0.5 mg/kg) based on results from a concurrent research project, which suggested the possibility of acute facilitation of stress-reactivity at a dose of 1 mg/kg of AM251 in loud noise stress. Rats were randomly divided into two groups (n = 6/group) and given i.p. injections of AM251 (0.5 mg/kg) or vehicle 30 minutes prior to a single restraint stress treatment (lasting 30 minutes). A blood sample was taken via tail nick immediately following restraint, and subsequent plasma CORT analysis supported 0.5 mg/kg AM251 to be without acute facilitating effect in restraint stress-reactivity. A similar pilot measure examining plasma CORT response to acute loud noise stress also supported this lower dose to be sub-threshold compared to vehicle treatment (n =3/group, data not shown). This additional lower dose was used, along with 1.0 mg/kg, in Experiment 3.

Rats (n = 53) were randomly assigned to receive repeated loud noise stress (n = 34) or to be used as acute stress controls for habituation testing (n = 19). Rats designated for repeated stress received 7 daily loud noise stress exposures (30 minute sessions, 95dBA) to allow for acquisition of homotypic stressor habituation. The remaining rats were handled and transported the same way as the repeatedly stressed rats, but were placed in quiet noise chambers for 30 minutes daily instead of receiving repeated loud noise stress. On day 8, the ability of CB1 receptor antagonism to reveal cross-sensitization of reactivity to acute restraint stress was tested. Repeatedly stressed rats were assigned to 3 groups (Vehicle, n = 11; 0.5 mg/kg AM251, n = 12; 1.0 mg/kg AM251, n = 11). Drug treatments were administered by i.p. injections 30 minutes prior to a 30 minute session of restraint stress. Blood samples were taken via tail nick immediately after stress treatment. We hypothesized that both doses of AM251 would reveal sensitized HPA axis responses in rats with recent repeated homotypic stress experience. On day 9, control rats were divided into two groups (Acute stress control/Vehicle, n = 9; Acute stress control/0.5 mg/kg AM251, n = 10), and all rats were exposed to loud noise stress with repeatedly stressed rats receiving the same drug pretreatments as day 8. Immediately following this stress treatment, rats were sacrificed by decapitation. Trunk blood was collected for CORT measurement, and brains were rapidly excised and frozen for later sectioning and immediate early gene measurements. We hypothesized that the 0.5 mg/kg AM251 treatment would not result in increases in stress-induced CORT in rats experiencing this stressor for the first time, but that both

doses of the CB1 receptor antagonist would lead to a higher response in rats that recently experienced repeated loud noise stress.

Acclimation

Animals were allowed two weeks of acclimation to the colony before testing. The first week, animals were housed in groups of three to four. During the second week of acclimation, rats were individually housed and handled daily, in the colony room, from days one through four. On each of the last three days before testing, rats were transported in their home cages from the colony to the testing room, handled, returned to their home cages, and placed inside individual acoustic chambers (without noise exposure) for thirty minutes. This pre-exposure was intended to familiarize the rats to all of the testing procedures and minimize novelty related responses on testing days.

Drug Treatment

The CB1 receptor antagonist AM251 (Ascent Scientific, Princeton, NJ) was used to assess the involvement of the endogenous cannabinoid system on plasma CORT and limbic c-*fos* mRNA responses to acute and repeated psychological stress. AM251 was dissolved in dimethyl sulfoxide (DMSO), Tween 80, and physiological (0.9%) saline (in a 1:1:8 ratio, respectively). A stir plate was used to maintain suspension of AM251 in vehicle, and syringes were loaded immediately prior to dosing. Rats received acute or repeated intraperitoneal (i.p.) injections in doses of AM251 at 2.0, 1.0, or 0.5 mg/kg, in injection volumes of 1 ml/kg. On drug treatment days, control rats received a similar

volume of vehicle (DMSO/Tween 80/0.9% saline) 30 minutes prior to placement in the acoustic chambers.

Loud Noise Stress

The acoustic chambers used in this experiment have been described in detail in Day et al. 2009. On the testing day, rats were placed in the acoustic chambers in their home cages thirty minutes after vehicle or AM251 injection. Rats were either kept under quiet “no noise” control conditions (background noise of fans approximately 57 dB SPL - A scale) or loud noise (95 dB) was turned on immediately and remained on for thirty minutes.

Restraint Stress

Restrainers were constructed from 0.64 cm wire mesh. The mesh was formed into 7.6 cm diameter cylinders that were 30.5 cm long. A 5.1 cm wide, 0.64 cm thick piece of white painted wood was placed at the bottom of the mesh cylinder to form a platform for the rat to sit on. The mesh was stapled to the wood on the outside of the cylinder. The ends of the cylinders were plugged with 7.6 cm diameter plastic atrium grates. Sections of the grates were removed to allow the rats' tails to protrude from the cylinders. The grates were secured on both sides of the restrainers with small bungee cords. With the grates in place, the internal dimensions of the wire mesh restrainers were similar to those of standard Plexiglas restrainers (17.8 cm length and 6.4 cm diameter) and have shown comparable stress-induced ACTH and CORT hormone

release (Masini et al., 2012). Restraint stress occurred in the same room as loud noise exposure after appropriate acclimation to testing procedures and location. Rats were removed from their cages immediately before being placed into restraint tubes, which were then placed on a lab bench covered in lab mat. Restraint tubes were cleaned with dish soap and hot water between uses.

Novel Environment Stress

Rats were placed in clean, white five gallon buckets (without bedding) for fifteen minutes as a mild novelty-related stressor (Babb et al., 2014). Buckets were cleaned with 50% ethanol and air-dried between uses to minimize odors.

Corticosterone Enzyme Linked ImmunoSorbent Assays (ELISA)

The corticoterone assay was performed according to the manufacturer's instructions (kit #K014-H5– Arbor Assays, Ann Arbor, MI) using 10 microliters of plasma. Levels were quantified on a BioTek Elx808 microplate reader and calculated against a standard curve generated concurrently.

In situ Hybridization

The method for *in situ* hybridization histochemistry has been previously described (Day and Akil, 1996). Briefly, 12 µm sections were cut on a cryostat (Leica model 1850), thaw- mounted on polylysine-coated slides and stored at –80°C. A [35S]- UTP-labeled riboprobe against c-*fos* mRNA (680 mer; courtesy of Dr. T. Curran, St Jude Children's

Hospital, Memphis TN) was generated using standard transcription methods. Sections were fixed in 4% paraformaldehyde (1 hour), acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride (10 min.) and dehydrated through graded alcohols. Sections were hybridized overnight at 55°C with a [35S]- UTP-labeled riboprobe diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 2× saline sodium citrate (SSC), 50 mM PBS, pH 7.4, 1× Denhardt's solution, and 0.1 mg/ml yeast tRNA. The following day, sections were treated with RNase A, 200 ug/ml at 37 °C (1 hour), and washed to a final stringency of 0.1× SSC at 65°C (1 hour). Dehydrated sections were exposed to X-ray film (BioMax MR; Eastman Kodak, Rochester, NY) for structure-appropriate times (1–3 weeks) and the films analyzed as described below. Structures chosen for *c-fos* mRNA analysis include regions with high levels of neuronal activity (as indicated by induction of *c-fos* mRNA) in response to acute loud noise stress (Burow et al., 2005) and regions displaying AM251-induced potentiation of noise stress-induced *c-fos* mRNA (Newsom et al., 2012). Also, the posterior hypothalamus was analyzed due to recent work in our lab implicating involvement of this region in habituation-related plasticity (Nyhuis et al., in preparation). The basolateral amygdala (BLA) was measured due to previous reports of repeated stress-related increases in eCB activity and levels (Hill, McLaughlin, et al., 2010a; Patel et al., 2005).

Semi-quantitative x-ray film analysis

Levels of c-*fos* mRNA were analyzed by computer-assisted optical densitometry. Anatomical landmarks were based on the white matter distribution of unstained tissue

sections, according to a standard rat brain atlas (Paxinos and Watson, 1998). Brain sections were captured digitally (CCD camera, model XC-77; Sony, Tokyo, Japan), and the relative optical density of the x-ray film was determined using Scion Image version 4.0 for PC. A macro was written (Dr. S. Campeau) that enabled signal above background to be determined automatically. For each section, a background sample was taken over an area of white matter, and a signal threshold was calculated as mean gray value of background + 3.5 standard deviation. The section was automatically density sliced at this value, so that only pixels with gray values above these criteria were included in the analysis.

Statistical Analyses

Prism (v 6.0, GraphPad Software Inc.) was used for all statistical analyses, which included two-way analyses of variance (ANOVA, repeated measures design when applicable), Bonferroni's multiple comparisons test for post-hoc analyses, and two-tailed t tests for pilot testing and planned comparisons, as indicated in the text. Significance for all tests was established at a $P = 0.05$. All data presented in the figures are listed as mean values +/– 1 standard error. Outlier values were identified as those being greater than 2 standard deviations from the group mean when included in the dataset, and were excluded. Additionally, some variation in degrees of freedom reflects sample loss during processing.

Results

Experiment 1: *Daily pre-injection of CB1 receptor antagonist AM251 before loud noise stress to examine role of eCB signaling in acquisition of the necessary habituation plasticity compared to the performance of habituation.*

Plasma CORT values from blood samples taken after 1, 3, 7, and 8 days of loud noise stress are presented in Figure 4.1, and were used to determine the effects of CB1 receptor antagonism on acquisition and expression of HPA axis habituation to repeated loud noise stress. Analysis of plasma CORT was performed with two-way repeated measures (RM) ANOVA with day of stress treatment (1,3,7,8) and drug treatment (vehicle, 0.5 mg/kg "low dose"AM251, and 2.0 mg/kg "high dose" AM251) as factors. A significant main effect of day ($F_{(3,28)} = 58.72$, $p < 0.001$) indicated significant habituation of HPA axis response to loud noise stress occurred in response to repeated exposures. Post hoc analyses using Bonferroni's multiple comparisons test confirmed that in all three groups, day 7 values of plasma CORT were significantly lower than day 1 values ($p < 0.05$) indicating that all treatment groups displayed significant habituation. A significant main effect of drug treatment ($F_{(2,56)} = 42.45$, $p < 0.001$) indicated that CB1 receptor antagonism altered plasma CORT values. Bonferroni's post hoc comparisons confirmed that high dose AM251 treatment significantly increased plasma CORT compared to the low dose and vehicle treatment groups ($p < 0.001$), which were not different from each other ($p > 0.05$). Significant interaction between drug treatment and day of stress treatment ($F_{(6,56)} = 4.58$, $p < 0.001$) indicated that daily CB1 receptor antagonism altered the measured habituation rate of HPA axis response to repeated

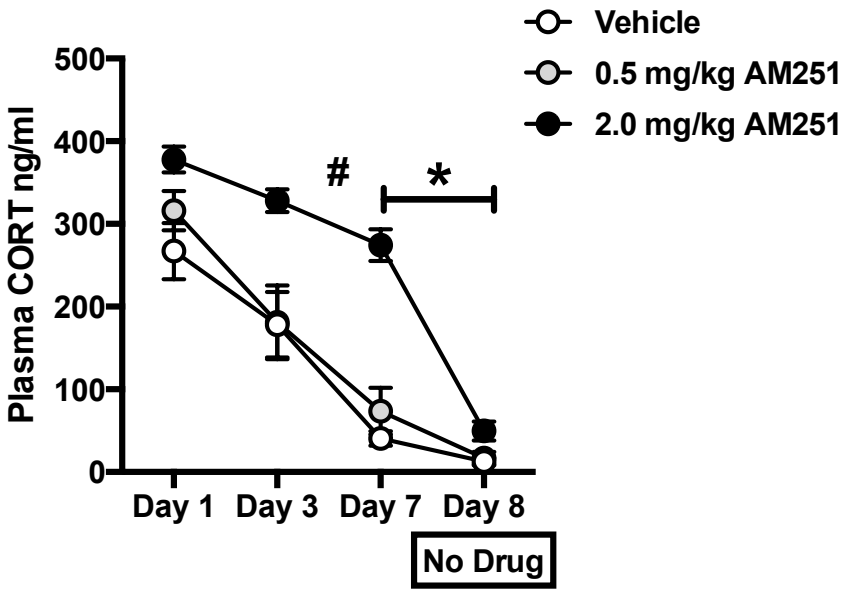

Figure 4.1 Dose-dependent ability of CB1 receptor antagonism to disrupt the expression of habituation but not the plasticity necessary for habituation of HPA axis response to repeated loud noise stress. Rats (n=8/group) were given systemic pre-treatment with CB1 receptor antagonist AM251 (2.0 or 0.5 mg/kg) or vehicle before each of the first 7 days of loud noise stress exposure (30 min/day, 95 dB). 2.0 mg/kg AM251 treatment significantly potentiated plasma corticosterone (CORT) responses and resulted in slower rate of habituation (# significant interaction of stress and drug, $p < 0.001$, confirmed post hoc with Bonferroni mct). Rats were not given drug treatment before the 8th loud noise stress exposure in which all three groups displayed similar level of HPA axis response habituation. The 2.0 mg/kg AM251 treatment group displayed significant reduction from day 7 to day 8, but 0.5 mg/kg AM251 and vehicle treatment groups did not (* $p < 0.001$).

loud noise stress. Bonferroni post hoc comparisons confirmed that high dose AM251 pretreatment resulted in insignificant reduction of HPA axis responses between days 3 and 7, compared to significant reduction between these time points in both low dose AM251 and vehicle treatments ($p < 0.05$). On the 8th day of loud noise exposure, rats were not given drug or vehicle pre-treatments to allow for testing for appropriate acquisition of the plasticity necessary for habituation. Bonferroni's post hoc comparisons used to examine interaction effects indicate that high dose AM251-treated rats demonstrated significantly greater reduction of plasma CORT response from day 7 to

day 8 ($p < 0.001$) compared to low dose and vehicle-treated rats, which did not display significant reductions between day 7 and day 8 ($p > 0.05$), and indicated no difference between the three groups on day 8 CORT values ($p > 0.05$). These analyses indicate that daily CB1 receptor antagonism has a dose dependent effect on initial and repeated HPA axis response to loud noise stress, such that 2 mg/kg AM251 pretreatments increased CORT responses to noise stress, and disrupted the expression of HPA axis habituation to repeated exposures, while not preventing the acquisition of the plasticity required for normal habituation. Further, these analyses indicate 0.5 mg/kg AM251 to be a dose without overt effect or in the acquisition or expression of habituated HPA axis responses.

Experiment 2: *Use of low dose of CB1 receptor antagonist AM251 pretreatment before 8th day of loud noise exposure and before heterotypic stressor exposure to assess CB1 receptor modulation of the expression of habituated responses and response sensitization to a mild, heterotypic stressor.*

Plasma CORT values from initial (day 1, non-treated) and repeated loud noise stress (day 8, drug or vehicle-treated) are presented with non-stress controls in Figure 4.2A. Two-way, RM ANOVA using day of stress exposure (day 1 or day 8) and drug treatment (vehicle, 1 mg/kg AM251) as factors indicated significant main effects of day of drug/stress treatment ($F_{(1,34)} = 17.52$, $p < 0.001$) and drug treatment ($F_{(1,15)} = 10.08$, $p < 0.01$), and a significant interaction between the two factors ($F_{(1,15)} = 11.71$, $p < 0.01$).

Bonferroni post hoc comparisons confirmed that rats administered vehicle injections on day 8 of loud noise stress displayed significantly habituated HPA axis responses compared to day 1 values ($p < 0.001$), but that 1 mg/kg AM251 pretreatment on day 8 resulted in plasma CORT level statistically indistinguishable from day 1 values

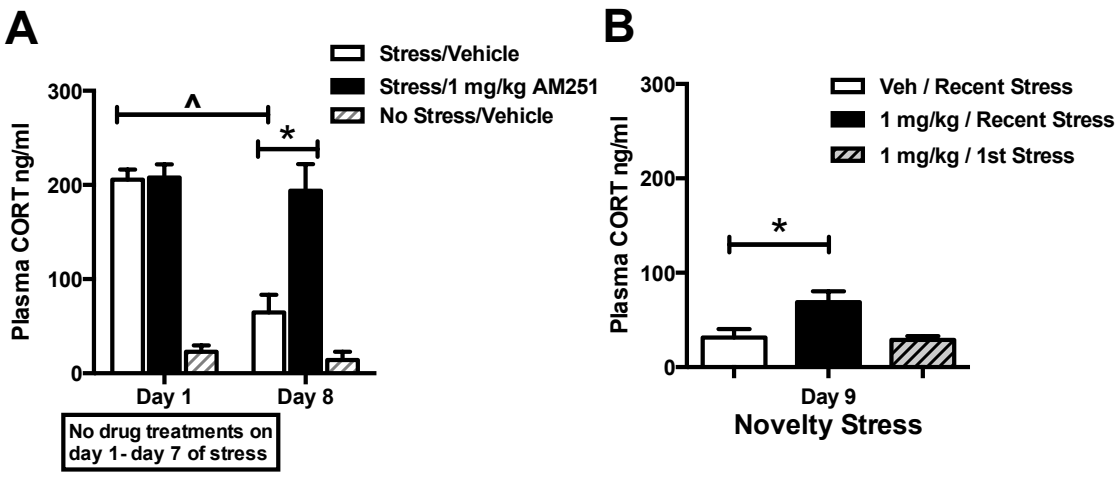

Figure 4.2 Low dose CB1 receptor antagonist AM251 (1 mg/kg) before 8th loud noise stress exposure prevents expression of HPA axis habituation. Evidence for heterotypic stressor sensitization. A. Rats (n=9/group) were exposed to daily loud noise stress without drug treatment (30 min/day, 95 dB). No stress control rats (n=3) included for day 9 control group. Pretreatment with 1.0 mg/kg AM251 before the 8th loud noise exposure completely prevented expression of a habituated HPA axis response. Vehicle-treated controls display robust habituation of plasma CORT response to the 8th loud noise exposure, compared to the 1st exposure (^ $p < 0.001$). 1 mg/kg AM251 treatment results in significantly higher plasma CORT response to the 8th loud noise exposure, compared to vehicle controls (* $p < 0.001$). **B.** Heterotypic stressor sensitization test: After 8 days of loud noise stress, rats were exposed to 15 minutes of novel environment stress on day 9. 1 mg/kg AM251 treatment significantly increased plasma CORT levels compared to vehicle-treated rats with the same stress history (* $p < 0.05$). A lack of facilitating effect on plasma CORT response to 1.0 mg/kg AM251 was observed in control rats without recent stress history when compared to their previous non-stress values or to vehicle treated rats with repeated stress history. However, the difference between the two 1.0 mg/kg-treated groups failed to reach significance ($p < 0.1$)

($p > 0.05$). Planned comparison of day 8 CORT values with two tailed t-test indicated that 1 mg/kg AM251 treatment significantly increased HPA axis response compared to the habituated response of vehicle treated controls ($T_{(16)} = 4.05$, $p < 0.001$), confirming

our hypothesis that CB1 receptor antagonism would disrupt the expression of habituation. An additional group of non-stressed control rats was placed in quiet noise chambers for 8 days during the repeated stress treatments. This group received the same acclimation and study-related handling as the stress-treated groups, including blood samples being taken on days 1 and 8 and i.p. vehicle injection on day 8. These values are included in the graph for visual comparison, but are not part of our experimental analysis. Mean plasma CORT values for these rats were 22.83 (+/- 6.74) and 14.04 (+/- 8.78) ng/ml on day 1 and day 8, respectively. Analysis by two-tailed, paired t test indicates these values to be statistically similar ($p > 0.05$). Plasma CORT response to the mild stress of a novel environment (Fig. 4.2B) was used to examine whether repeated noise stress experience would result in a cross-sensitization to heterotypic stress that may be dependent on disruption of CB1 receptor signaling. Control rats were included for examination of acute effects (without recent repeated stress history) of 1 mg/kg AM251 on reactivity to the novel environment. A one-way ANOVA indicated a significant difference among treatment groups ($F_{(2,17)} = 4.43$, $p < 0.05$). Bonferroni post hoc analysis indicated that 1 mg/kg AM251 treatment before novel environment stress resulted in higher plasma CORT levels in rats with repeated noise stress experience compared to vehicle-treated controls with the same stress history ($p < 0.05$), offering evidence for stressor cross-sensitization mediated by eCB system disruption. However, in the same post hoc analysis, plasma CORT responses in vehicle-treated rats without recent stress history were not found to statistically differ from either treatment group ($p > 0.05$). Comparisons of the two previous Day 8

treatments within each group found no effect of previous drug treatment on day 9 CORT level (t test, $p = 0.64, 0.73$; data not shown).

Experiment 3: *Examination of generalizability of repeated stress-related cross-sensitization to restraint stress, and comparison of stress-dependent neural activity of CB1 receptor antagonism in acute and habituated stress.*

We added a lower dose (0.5 mg/kg) of AM251 treatment group in Experiment 3 to ensure that any stimulatory effects of AM251 observed after repeated stress were independent of acute potentiation from CB1 receptor antagonism. Pilot testing supported a lack of potentiating effect of 0.5 mg/kg AM251 in acute restraint stress ($p = 0.75$; Fig. 4.3D) as did the results of experiment 1 earlier in this chapter. Habituation to repeated loud noise stress was analyzed by comparing plasma CORT levels from the 1st and 8th loud noise exposure (Figure 4.3). Two-way RM ANOVA with stress treatment (acute vs. repeated) and drug treatment (vehicle, 0.5, and 1.0 mg/kg AM251) indicated significant main effects of stress ($F_{(1,28)} = 71.4$, $p < 0.001$), but not drug treatment ($p > 0.05$), and a significant interaction between the two ($F_{(2,28)} = 5.24$, $p < 0.05$). Bonferroni post hoc comparisons confirmed that rats receiving vehicle treatment and 0.5 mg/kg AM251 displayed significantly habituated plasma CORT levels compared to non-treated day 1 values ($p < 0.001$), however 1.0 mg/kg treatment before the 8th noise exposure resulted in statistically similar values to day 1 ($p > 0.05$; Fig. 4.3A). Planned

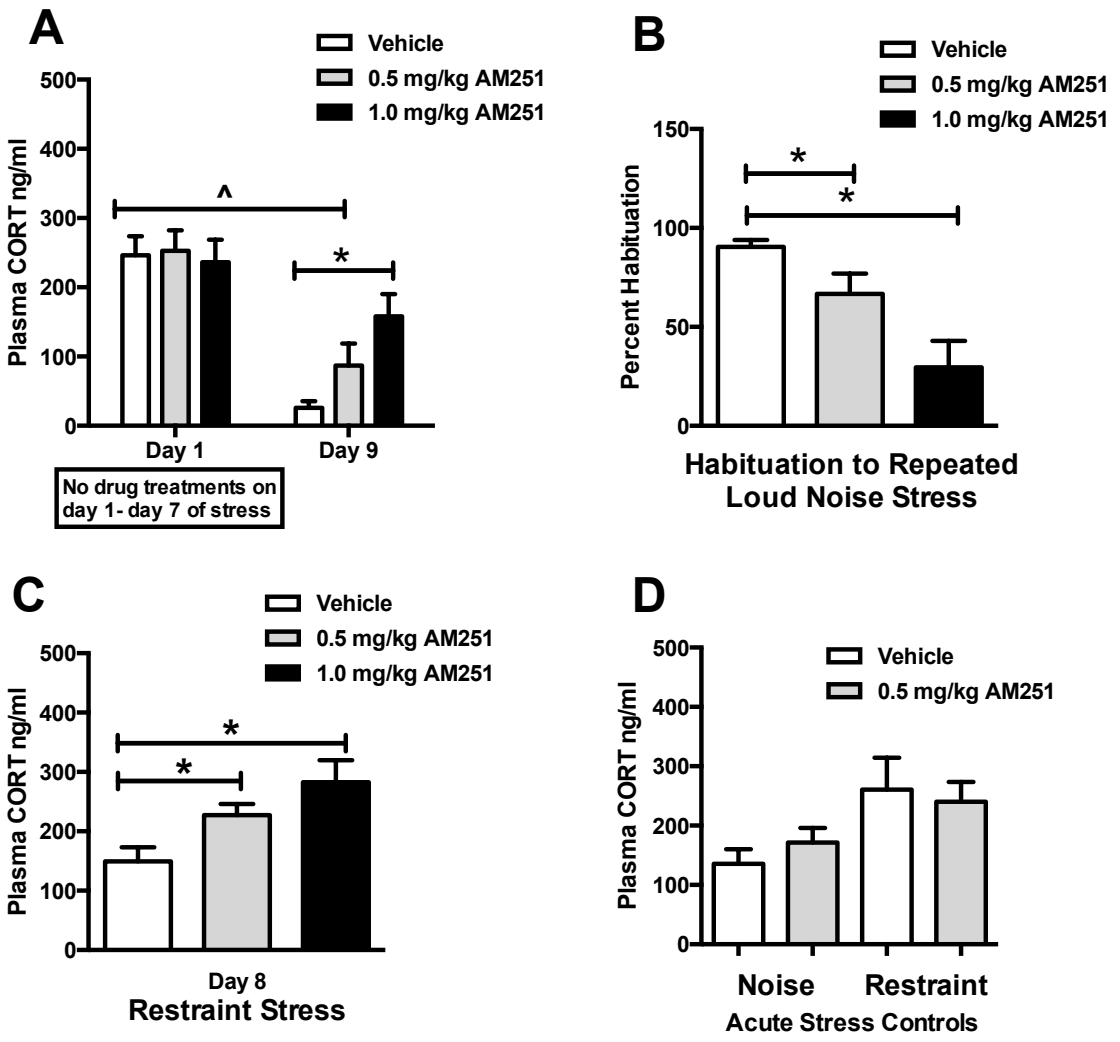

Figure 4.3 0.5 and 1.0 mg/kg AM251 disrupt the expression of habituation to loud noise stress, and result in sensitized HPA axis response to a heterotypic stressor. A. Vehicle and 0.5 mg/kg AM251 treatment groups display significant habituation on Day 9 (^ $p < 0.05$). 1.0 mg/kg AM251 significantly reduced the expression of habituation on Day 9 (* $p < 0.05$). 0.5 mg/kg treatment did not reach significance in this measure ($p = 0.1$). **B.** Plasma CORT response to repeated noise stress graphed as percent of Day 1 response. Both doses of AM251 significantly reduced the percentage of habituation, indicating a disruption of the expression of habituation (* $p < 0.05$). **C.** Day 8 sensitization testing. Repeatedly stressed rats displayed significantly increased plasma CORT response to a heterotypic stressor, (* $p < 0.05$). **D.** Acute noise and restraint stress controls. 0.5 mg/kg AM251 does not potentiate plasma CORT response to restraint stress in rats without recent repeated stress history (pilot). 0.5 mg/kg AM 251 does not potentiate plasma CORT response to the initial loud noise exposure in acute stress control rats. Tissue was collected from rats after acute noise stress for comparisons of neural activity to repeatedly stressed rats.

comparisons of vehicle-treated rats to 0.5 and 1.0 mg/kg AM251 treatment groups with independent two-tailed t test indicated that 1.0 mg/kg of the CB1 receptor antagonist significantly increased plasma CORT levels compared to vehicle-treated controls ($T_{(19)} = 3.78$, $p < 0.001$), but a similar pattern resulting from 0.5 mg/kg treatment did not reach significance ($p = 0.1$). However, compared to vehicle controls, both doses of AM251 were found to result in significantly lower percentage of habituated plasma CORT responses compared to each rat's acute, day 1 response to noise stress (0.5 mg/kg: $T_{(19)} = 2.10$, $p < 0.05$; 1.0 mg/kg: $T_{(19)} = 4.22$, $p < 0.001$; Fig. 4.3B). Two additional groups of rats were exposed to acute loud noise stress on day 9 of experiment 3 after pretreatment with 0.5 mg/kg AM251 or vehicle. Tissue from these acutely stressed rats was used for comparison in neural measures. These treatments were found to result in similar plasma CORT values when compared with two-tailed t test ($p = 0.34$; Fig.4.3D). Cross-sensitization to restraint stress was tested on day 8 of experiment 3 (Fig. 4.3C). One-way ANOVA indicated a significant difference in stress-induced plasma CORT levels ($F_{(2,28)} = 5.68$, $p < 0.01$). Planned comparisons between vehicle-treated rats and rats treated with 0.5 or 1.0 mg/kg AM251 with independent two-tailed t tests confirmed that both doses of AM251 resulted in facilitated HPA axis responses compared to vehicle treatment (0.5 mg/kg: $T_{(18)} = 2.58$, $p < 0.05$; 1.0 mg/kg: $T_{(19)} = 2.98$, $p < 0.01$).

Stress-induced c-*fos* mRNA was measured in tissue from rats administered 0.5 mg/kg AM251 or vehicle before acute or repeated loud noise. We hypothesized that 0.5 mg/kg of CB1 receptor antagonist AM251 would not result in increases in c-*fos* mRNA compared to tissue from vehicle-treated rats after acute loud noise stress, but would in

tissue collected from rats given repeated loud noise stress. This pattern was measured in several hypothalamic and extra-hypothalamic limbic structures able to modulate stress-reactivity. Data for all regions were analyzed with two-way ANOVA, post hoc Bonferroni multiple comparisons, and independent two-tailed t test for planned comparison between vehicle and 0.5 mg/kg AM251 treatment in repeatedly stressed rats. Consistent with the majority of HPA axis measures in this experiment, 0.5 mg/kg AM251 was determined by post hoc analyses to not alter c-*fos* mRNA induction by acute noise stress treatment ($p > 0.05$). This was consistent in all neural regions examined. Values for all c-*fos* mRNA analyses are graphed as a percent of the average value for control (acute stress, vehicle-treatment) rats in Figures 4.4, and 4.5.

The paraventricular nucleus of the hypothalamus (PVN) was measured to display a pattern similar to plasma CORT values in rats treated with 0.5 mg/kg AM251, such that this dose resulted in significant increase in c-*fos* mRNA in rats with repeated noise stress history, but not in acutely stressed rats (Fig. 4.4A). Two-way ANOVA with stress treatment (acute vs. repeated) and drug treatment (vehicle or 0.5 mg/kg AM251) as factors indicated a significant main effect of stress treatment ($F_{(1,38)} = 27.55$, $p < 0.001$), but not overall drug treatment ($p > 0.05$) or interaction between the two treatments ($p > 0.05$). Bonferroni post hoc analysis confirmed that c-*fos* mRNA values were significantly decreased after the 8th exposure to loud noise stress compared to values measured in acute rats, in both vehicle ($p < 0.001$) and AM251 ($p < 0.05$) treatment groups, and that AM251 treatment was without effect in acutely stressed rats ($p > 0.05$). Planned

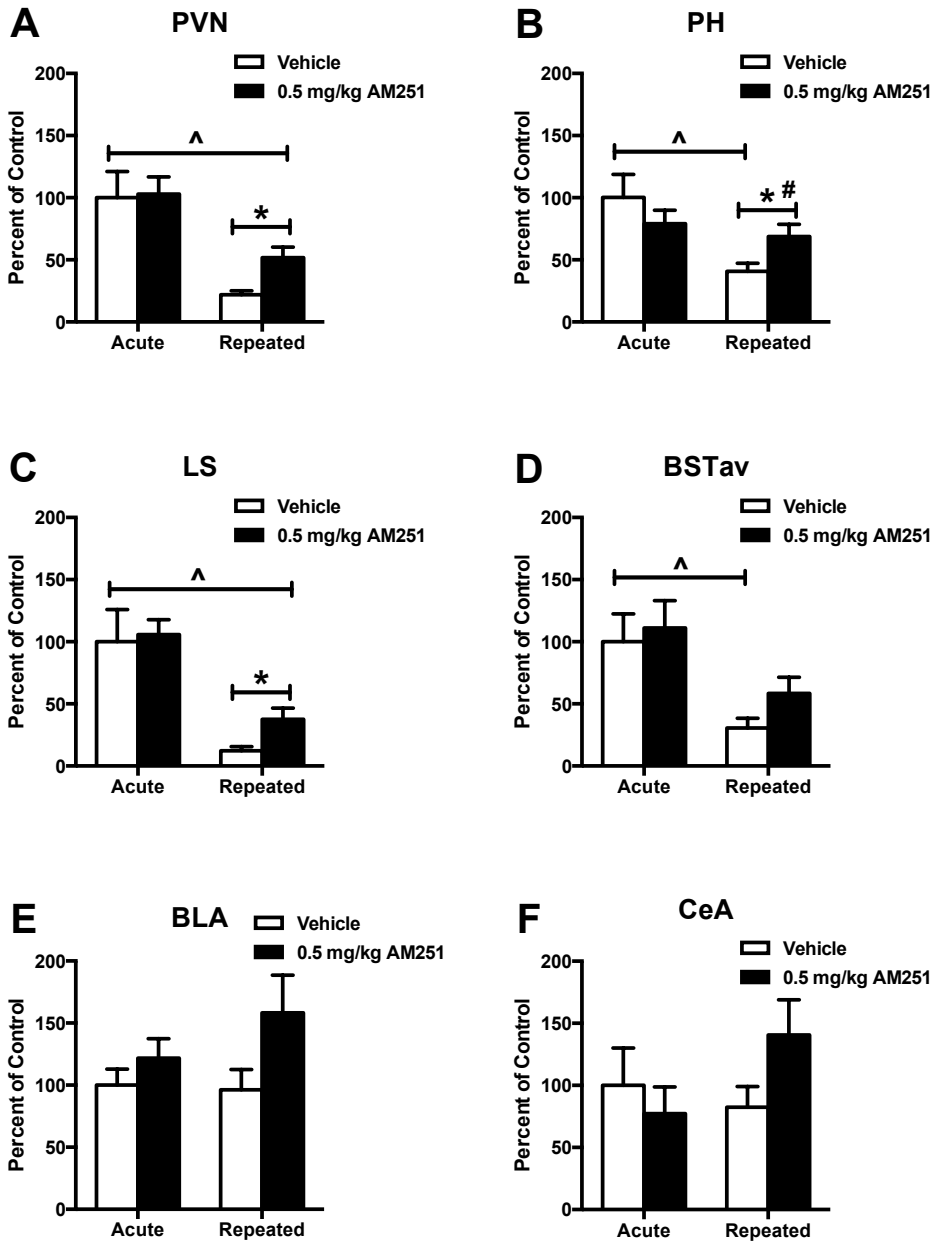

Figure 4.4 CB1 receptor antagonism disrupts the habituation of neural activity in multiple limbic regions. Stress-induced c-*fos* mRNA significantly habituated in the PVN, PH, LS, and BSTav after 8 exposures to repeated loud noise stress (^ $p < 0.05$). 0.5 mg/kg AM251 did not significantly increase c-*fos* mRNA responses to initial, acute loud noise stress, but this dose significantly disrupted the expression of c-*fos* mRNA habituation in the PVN, PH, and LS (* $p < 0.05$ planned t test comparisons). A significant interaction of drug and stress experience was measured in the PH (# $p < 0.05$). BLA and CeA c-*fos* mRNA did not display significant increase by CB1 receptor antagonism.

comparison indicated that in repeatedly stressed rats, 0.5 mg/kg AM251 treatment increased PVN c-*fos* mRNA compared to vehicle treatment ($T_{(21)}$ = 3.14, $p < 0.01$), supporting involvement of a central eCB mechanism in the disrupted expression of HPA axis habituation.

Posterior hypothalamus (PH) analysis indicated a similar pattern of CB1 antagonist-mediated disruption of habituation in repeatedly stressed rats, with AM251 treatment resulting in complete restoration of c-*fos* mRNA induction to levels measured in rats treated with AM251 before acute stress (Fig. 4.4B). Two-way ANOVA indicated a significant main effect of stress treatment ($F_{(1,37)}$ = 9.06, $p < 0.01$) and a significant interaction between drug and stress treatments ($F_{(1,37)}$ = 4.49, $p < 0.05$). Post hoc analysis determined that c-*fos* mRNA induction was significantly habituated on the 8th exposure to loud noise stress compared to the first exposure in vehicle-treated rats ($p < 0.01$). AM251 treatment before the 8th noise stress exposure significantly increased c-*fos* mRNA values compared to vehicle-treated controls ($T_{(20)}$ = 2.41, $p < 0.01$). Analysis of lateral septum (LS, graph C) c-*fos* mRNA induction with 2-way ANOVA revealed significant main effect of stress treatment ($F_{(1,35)}$ = 29.05, $p < 0.001$), but not drug ($F_{(1,35)}$ = 1.14, $p = 0.29$), or interaction between the two ($F_{(1,35)}$ = .46, $p = 0.50$). Bonferroni post hoc comparisons confirmed that both AM251 and vehicle-treated rats displayed significantly habituated c-*fos* mRNA values after the 8th day of noise stress compared to acute stress controls ($p < 0.01$). As measured in the PVN and PH, 0.5 mg/kg AM251 treatment significantly increased c-*fos* mRNA in the LS on the 8th day of loud noise stress compared to vehicle treatment ($T_{(19)}$ = 2.32, $p < 0.05$). A similar pattern in the

anterior ventral bed nucleus of the stria terminalis (BSTav, Fig. 4.4D) failed to reach significance in t test comparison ($p = 0.1$). In this region, two-way ANOVA revealed a significant effect of stress treatment ($F_{(1,37)} = 13.04$, $p < 0.001$) that was confirmed by post hoc comparisons to indicate that in vehicle-treated rats, repeated noise stress resulted in significantly reduced c-*fos* mRNA induction compared to acute noise stress ($p < 0.05$). Interestingly, in the same post hoc comparisons, BSTav c-*fos* mRNA induction was not found to significantly differ between acute and repeated stress conditions ($p > 0.05$).

Analysis of basolateral amygdala (BLA) c-*fos* mRNA with 2-way ANOVA indicated no significant differences due to stress ($F_{(1,36)} = 0.53$, $p = 0.47$) drug ($F_{(1,36)} = 3.44$, $p = 0.07$), or stress x drug interaction ($F_{(1,36)} = 1.79$, $p = 0.38$; Figure 4.4E). Planned comparison of BLA c-*fos* mRNA in repeatedly stressed rats found no difference due to drug treatment ($T_{(21)} = 1.74$, $p = 0.1$). Similarly, central amygdala (CeA) c-*fos* mRNA values (Fig. 4.4F) were not found to differ due to stress ($F_{(1,34)} = 0.39$, $p = 0.53$) drug ($F_{(1,34)} = 0.15$, $p = 0.70$), or interaction of stress and drug treatments ($F_{(1,34)} = 2.02$, $p = 0.16$). Planned comparison of vehicle and AM251 treatments in repeatedly stressed rats did not indicate a difference in CeA c-*fos* mRNA ($T_{(17)} = 1.38$, $p = 0.18$).

Cortical c-*fos* mRNA was measured in several stress-related regions, and can be found in Figure 4.5. In general, this marker of neural activity was found to habituate in the auditory (AUD) and infralimbic (IL) regions, but not in the orbitofrontal (OFC) or prelimbic (PL) regions. None of the four regions displayed significant effect of CB1 receptor antagonism in acute or repeated stress conditions. In AUD cortex c-fos mRNA,

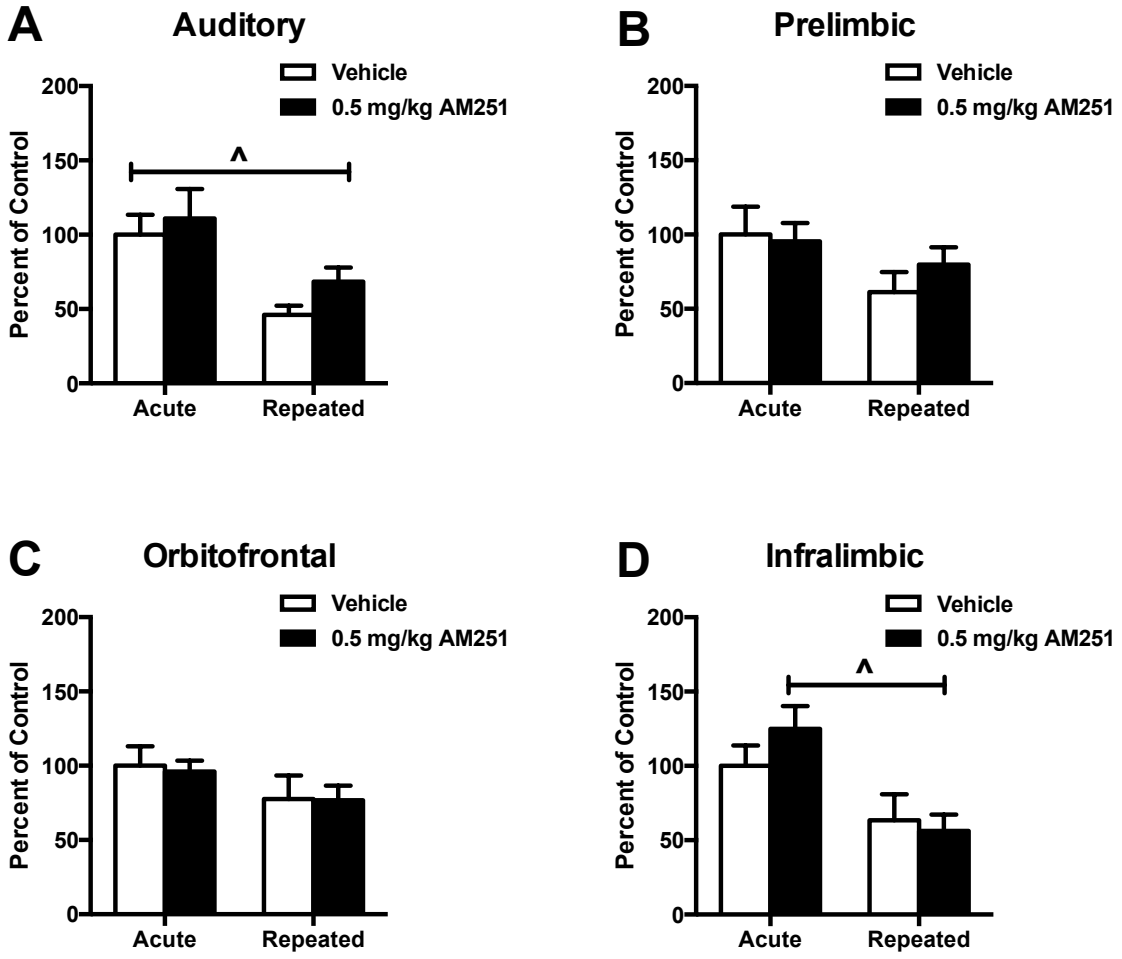

Figure 4.5. Cortical c-*fos* mRNA in Auditory cortex (**A**) and infralimbic cortex (B) c-*fos* mRNA was measured to significantly habituate after 8 loud noise stress exposures (^ $p < 0.001$). Prelimbic (**B**) and orbitofrontal (**C**) cortex c-*fos* mRNA did not significantly habituate to repeated loud noise stress.

2-way ANOVA revealed significant effect of stress ($F_{(1,36)} = 15.04$, $p < 0.001$), but not drug treatment ($F_{(1,36)} = 1.78$, $p = 0.19$), or stress x drug interaction ($F_{(1,36)} = 0.21$, $p = 0.65$; Fig. 4.5A). Bonferroni multiple post hoc comparisons indicated significant habituation in repeatedly stressed, vehicle-treated rats compared to acute stress controls ($p < 0.05$), but this difference was not significant in AM251 treatment groups (p

> 0.05). Planned comparison of AM251 to vehicle treatment did not find a difference in c-*fos* mRNA expression on the 8th day of loud noise stress ($T_{(20)}$ = 1.97, p = 0.06). Analysis of PL and OFC c-*fos* mRNA revealed no effects of stress treatment (PL: $F_{(1,35)}$ = 3.83, p = 0.06; OFC: $F_{(1,38)}$ = 2.99, p = 0.09), drug treatment (PL: $F_{(1,35)}$ = 0.25, p = 0.62; OFC: $F_{(1,38)}$ = 0.04, p = 0.84), or stress x drug interaction (PL: $F_{(1,35)}$ = 0.69, p = 0.41, OFC: $F_{(1,38)}$ = 0.02, p = 0.90; Fig. 4.5 B,C). Planned comparisons in repeated stress groups for both regions did not indicate difference of drug treatment (PL: $T_{(19)}$ = 1.03, p = 0.31; OFC: $T_{(21)}$ = 0.04, p = 0.97). Analysis of IL c-*fos* mRNA with 2-way ANOVA revealed significant effect of stress treatment ($F_{(1,37)}$ = 12.82, p = 0.001) but not drug treatment ($F_{(1,37)}$ = 0.37, p = 0.55) or interaction between the two ($F_{(1,37)}$ = 1.19, p = 0.28; Fig. 4.5D). Planned comparison in repeatedly stressed rats indicated no difference attributable to drug treatment in this region ($T_{(20)}$ = 0.35, p = 0.73).

Discussion

The results of these studies are in agreement with the involvement of inhibitory endogenous cannabinoid signaling in both the reduction of neural and HPA axis responses to repeated homotypic stress characteristic of habituation, as well as a type of heterotypic stressor sensitization related to neuroplastic alterations resulting from repeated stress experience, which requires mild disruption of neural signaling at CB1 receptors to manifest. It is currently unclear to what extent these two response patterns are related. Additionally, our results indicate an important distinction of inhibitory eCB system influence on the expression of habituated responding to stress, but not as a

necessary component of the plasticity involved in the acquisition of habituation. Multiple neural regions were found to display increased c-*fos* mRNA in a pattern indicating a multi-structural consequence of disrupting the increase(s) in, or recruitment of, CB1 receptor signaling that was measured in rats exposed to repeated noise stress, but not in rats without recent repeated stress history. This pattern is importantly distinguishable from the more widespread potentiation of c-*fos* mRNA that we have found to occur with higher dose AM251 administration before an initial experience of loud noise stress (Newsom et al., 2012) and offers evidence in support of a multi-structural circuitry involved in the inhibition of neuroendocrine responses to familiar, innocuous stressors.

CB1 receptor involvement in habituation of HPA axis response to repeated noise stress.

In Experiment 1, daily administration of an acutely potentiating dose (2.0 mg/kg) of CB1 receptor antagonist resulted in increased CORT in blood samples taken immediately after stress treatment on days 1, 3, and 7, and an inhibited habituation rate to repeated loud noise stress exposures compared to vehicle controls and a lower dose (0.5 mg/kg) of AM251. However, by the 7th loud noise exposure with drug pretreatment, a significant habituation of plasma CORT level was measured compared to the day 1 values for the same group. A final loud noise exposure on day 8 without drug pretreatment revealed that this group displayed a level of habituation that was indistinguishable from the other two groups, indicating that repeated AM251 treatment

did not reliably interfere with the development of the neural alterations necessary for the predictable habituation to this intensity of noise.

We have previously found this dose of AM251 to robustly potentiate limbic and neuroendocrine reactivity to loud noise stress, as well as to induce significant CORT elevation and activity in various discreet neural regions in the absence of stress (Newsom et al., 2012). A potential alternative interpretation for the pattern of plasma CORT measured on days 1, 3, and 7 could be that instead of indicating disruption of a central habituation-related mechanism, this pattern simply reflects an additive contribution of direct pharmacological adrenal gland stimulation that can occur with this dose of AM251. However, this interpretation cannot easily account for the altered rate of habituation, and strong evidence from our lab and others indicates a widespread, multifaceted ability of central eCB signaling to negatively modulate HPA axis reactivity to acute stress. To distinguish the involvement of eCB signaling in habituation and sensitization from the ability of CB1 receptor antagonism to directly stimulate or potentiate neuroendocrine activities, we utilized two lower doses of AM251 with limited acute activity in our measures (as in: Hill et al., 2010). Inverse agonist-like effects of AM251 and similar CB1 receptor antagonist SR141716A (Rimonabant) on neuroendocrine activity are not fully understood, but we have demonstrated them to be specific to certain neural regions rather than uniformly present in the brain, and to be positively related to dose (Newsom et al., 2012; Patel, 2004a). A recent study in our lab indicates systemic administration of 1.0 mg/kg AM251 does not stimulate CORT elevation or c-*fos* mRNA induction in the absence of stress. The results of our studies

with loud noise exposure and the different AM251 doses are highly consistent across studies and across laboratories (Hill, McLaughlin, et al., 2010a; Newsom et al., 2012).

In Experiment 2, 1.0 mg/kg AM251 administration before the 8th loud noise exposure resulted in total restoration of plasma CORT values to the levels measured after the first presentation. A control group without recent stress history was given this same dose before novel environment stress on the 9th day. Though plasma CORT values for this group were not found to be significantly lower than the repeatedly stressed rats receiving the same dose, an informal comparison indicates them to be similar to the non-stressed values of CORT measured day 1 and 8 from the same rats, supporting a sub-threshold effect of 1.0 mg/kg AM251 with mild stress. It appears likely that the effects of low dose AM251 depend on aspects such as stressor intensity and normal or intentional variability in testing environment and paradigm (Dallman et al., 1999; Gamble-George et al., 2013; Moreira & Wotjak, 2010).

The results of this study suggest that repeated stress experience alters central eCB activity involving CB1 receptors in a way to increase sensitivity to CB1 receptor antagonism in psychological stress. We observed this mechanism to disrupt the expression of habituation, and to result in novel stressor sensitization. It is unclear if these are distinct mechanisms, or if a single repeated stress-induced neuroplastic alteration is able to increase neuroendocrine responsivity to familiar and novel stressors. It is difficult to compare the extent of HPA axis increases we observed between the novel and familiar stressors because many neuroendocrine regions are more dynamically sensitive to mild stressors than to higher intensity stressors, which

may approach a ceiling of responsiveness. This distinction may not be of importance for clinical populations, in which the increased sensitivity to stress would have the same impact on psychoneuroendocrine reactivity regardless of the neural mechanisms involved.

We measured repeated stress-related increases in immediate early gene c-*fos* mRNA induction in several neural regions that was not observed in rats that were given the same dose of AM251 (0.5 mg/kg) before their initial acute loud noise stress exposure. This suggests that the facilitation we measured in HPA axis activity is not entirely the result of adrenal or pituitary gland alterations. The amygdala, lateral septum, and bed nucleus of the stria terminalis are all able to modulate neuroendocrine reactivity (Herman et al., 2005), making it plausible that the increases we measured are indicative of disrupting the activity of a multi-structured limbic mechanism responsible for the expression of stress habituation. If this were the case, it would be possible that the CB1 receptor-related disruption occurred in a single primary region and resulted in secondary excitation of other regions. Also, the presynaptic location of CB1 receptors supports a possibility that the limbic facilitation occurred due to disruption of CB1 receptor signaling at the synapses of afferent projections to these regions from a more removed structure that is of primary importance in habituation (Ramikie et al., 2014).

Chapter 5

1.

Proposal for support to measure the effects of local blockade of CB1 receptors on basal and stress-related pituitary and adrenal gland activity in male rats.

My doctoral research has focused on a role of the endogenous cannabinoid (eCB) system in regulating neural and hormonal activity in response to acute stress. This research has implications for stress-induced development of widely experienced disorders such as anxiety, depression, and post-traumatic stress disorder (PTSD), as well as general declines in physical and psychological wellbeing due to chronic stress. My research suggests that the eCB system is generally protective in the brain and body, and that decreased functioning of this system would result in a multifaceted state of vulnerability. My ultimate goal is to explore and develop dietary and (non-psychoactive) pharmacological strategies to protect the eCB system in the face of modern, stressful life. This exploratory idea is a little ahead of its time, and funding sources for this type of work are limited. Completion of the project in this proposal will not only contribute to my graduate development and wrap up my dissertation researches, it will help back up an important aspect of the philosophy of health that I am developing. I will have a more complete research story to use in pursuing future funding.

The eCB system is widely expressed in the brain and body and includes locally produced (endogenous) chemical signals and their target receptors, which exert a temporary and mild inhibition of cellular activity when signaled. Interestingly, the mild

cellular inhibition of eCB signaling is usually induced by the very cellular activity that is ultimately decreased or turned off by this system (3). This pattern has led to the eCB system being understood to function as a negative feedback system in a variety of cell populations in the brain and body. I hypothesized that the eCB system could function as an important “inhibitory buffer” of the cellular activity that collectively contributes to our responses to modern psychological stressors such as work-related demands, urban commuting (traffic) or uncertainty.

I have since demonstrated that pharmacological blockade of CB1 receptors before a psychologically stressful experience results in a potentiation of several responses that collectively contribute to the psychological and physical reaction commonly known as the “fight or flight response.”(8) This response pattern includes the coordinated activation of several neural (brain) areas and bodily systems in a manner that increases psychological arousal and provides a burst of energy to assist the organism in meeting the demands of a threatening situation (1). While temporary activation of this system can be protective, excessive (prolonged, repeated, or exaggerated) activation of this system is damaging, and can contribute to a wide variety of psychological and physical disorders (5,6,7). My initial research suggested that in a state of normal, healthy functioning, eCB signaling reduces or buffers the reaction of parts of the brain involved in detecting and reacting to stressors, and limits activity in hormonal systems responsible for the stimulatory stress-hormone cortisol. My results were in agreement with a view of the eCB system as a protective system in the brain

and body that, by buffering multiple responses to stressors, can reduce the accumulation of the negative consequences of repeated stress.

An unexpected finding in my initial study offered an interesting possibility of expanding the understanding of the actions of the eCB system. My proposal relates to this finding.

A control group in the experiment was treated with pharmacological blockade of CB1 receptors but was not exposed to the psychological stressor. Measures of neural and hormonal activity suggested that blockade of inhibitory CB1 receptors alone was capable of inducing activity in some brain regions of interest, as well as an increase in corticosterone (CORT, the rodent equivalent of stress hormone cortisol) in the bloodstream. This pattern was in stark contrast to other stress-reactive areas of the brain and hormone system which were not aroused to action by CB1 receptor blockade alone, but rather were found to exhibit a potentiation of stress-induced activation (which fits with my hypothesized disruption of the known actions of eCB signaling as a negative feedback mechanism). Of particular interest was the CB1 receptor blockade-induced stimulation of activity in a brain region (the amygdala) responsible for psychological fear responses, which is also known to be hyperactive in human anxiety, depression, and PTSD (2,4,11,12). The stimulation of neural activity in the amygdala that resulted from pharmacological blockade of CB1 receptors suggests that the eCB system functioned differently in this structure than in most others. I interpret this finding to suggest that in the amygdala, there is a constant activity of eCB signals at CB1 receptors, which inhibits activity of the structure. This constitutive “inhibitory tone” mechanism of the eCB system is distinct from the well-known negative feedback actions of the eCB system that

require stimulation to be expressed. We have since replicated this structural distinction of eCB activity and ruled out the influence of the minor stress of the drug administration procedure (systemic injection with a needle and syringe) on the stimulation of activity in the amygdala by remotely administering the CB1 receptor-blocking drug. This method of remote administration requires a minor surgery to implant a catheter in the rats to allow for undetected injection of the drug through a flexible tube. This method will be used in the proposed work.

***As above, so below* (?)**

My proposal is to examine the possibility that the same two distinct patterns of eCB activity (tonic inhibition of activity and negative feedback buffering of stress-induced stimulation) are not only present in the brain, but also contribute to the regulation of cortisol release directly at the level of the adrenal gland. If true, this finding would have exciting therapeutic implications. Chronic exposure to elevated levels of cortisol (which is produced by the adrenal glands and released into the bloodstream as part of the fight or flight response to stress) is thought to be a main contributing factor in stress-related development of psychological disorders and physical disease states (9). However, cortisol is also extremely important for maintaining the health of the brain and various aspects of the body including memory systems, energy balance, sleep/wake cycle, and regulation of inflammation (10,13). In the absence of stress, cortisol levels in the body follow a predictable daily pattern of an elevation that peaks around the time of awakening, and steady decline to the point of absence in the bloodstream in the hours before bed. During sleep, the elevation begins again. (13) Both the peak and the

absence of cortisol appear to be important in coordinating circadian (24 hr) rhythm patterns such as the sleep/wake cycle, as well as supporting the regulation of energy balance, cognitive function, and regulation of inflammation. I suspect that the eCB system exerts a constant, tonic inhibition of activity in the adrenal gland that is necessary for the complete lack of cortisol that lasts for a significant portion of the circadian cycle of adrenal activity. I also suspect that stress-induced stimulation of cortisol is buffered by the negative feedback mechanism of eCB activity directly in adrenal tissue as psychological reaction to stress is buffered by eCB activity in the brain.

In our previous studies, we used a drug that travels freely through the body to block CB1 receptors inside and outside the brain. As the brain ultimately regulates the hormonal activity of the body, I am not yet able to rule out the influence of brain activity on the stimulation patterns of CORT that we measured. In the initial studies (which were performed during the period of absence of CORT in the circadian cycle), we found CB1 receptor blockade to significantly increase CORT levels in the bloodstream in the absence of stress, while failing to show any sign of increase of pituitary gland activity (which would be expected if the increase in CORT was due to stimulation of brain areas able to influence the adrenal gland. The pituitary gland is a necessary mediator between the brain and adrenal gland.). The clear absence of activity of the pituitary gland was observed in a marker of cellular activity as well as the pituitary hormone ACTH, which is the only known direct stimulator of adrenal production of CORT. This result supported an idea that the eCB system enacts a steady, tonic inhibition of the adrenal gland, and

that the elevation in CORT was due to disruption of this mechanism by blockade of CB1 receptors. We have confirmed the presence of CB1 receptors in the adrenal gland via mRNA measure. Evidence of eCB involvement in buffering of stress-induced stimulation was observed in both studies but cannot yet be distinguished from the influence of eCB activity in the brain. We have found systemic blockade of CB1 receptors to result in potentiated blood levels of CORT in rats exposed to psychological stress, but we have also measured potentiation of stress-induced pituitary hormone ACTH and a marker of cellular activity. Potentiation of pituitary gland responses to stress would be expected if the potentiated hormone activity resulted from disruption of CB1 receptor inhibition in the brain. I am unable to distinguish the effects of local adrenal gland eCB system activity from eCB activity in the brain in my current data sets. To explore the actions of the eCB system in adrenal gland tissue, I would like to use a drug that can block CB1 receptors in the body, but is unable to cross into the brain. I expect that remote administration of this drug alone will elevate blood levels of CORT and increase markers of activity in adrenal tissue without increasing hormonal or cellular activity in the pituitary gland. I expect that a combination of CB1 receptor blockade and psychological stress will result in potentiation of stress-induced stimulation of CORT and adrenal cellular activity, without resulting in potentiation of pituitary gland activity, as measured by blood level of ACTH and markers of cellular activity.

Steps of the proposed research project:

-Surgical implantation of catheter to allow for remote administration of CB1 receptor blocking drug that cannot cross into the brain. I will allow 10-14 days for recovery from surgery before testing.

-Testing day: Administer drug or vehicle remotely to rats 30 minutes before exposure to psychological stress or non-stress control treatment. Stress treatment to last 30 minutes. Research design: 2x2 between subjects

- 4 groups of rats with n=10 in each. Groups: drug/stress, drug/no stress, vehicle/stress, vehicle/no stress

After stress treatment: rats will be rapidly sacrificed, trunk blood will be taken for subsequent measure of hormones ACTH and CORT, adrenal and pituitary glands will be harvested and rapidly frozen for later sectioning and measure of activity markers (including c*fos* mRNA).

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2.

This is part of a draft I pitched to one of my mentors for a grant proposal. We had a great time thinking together, and back and forth, then holding our breath while we waited to hear back about the funding decisions. He reeled me in to make it a better grant ("Ryan, sometimes less is more."), and taught me all along the way. Certainly one of my best experiences in the program.

It's all in here. *I am including this in my dissertation as a memento and a trophy considering what the final submission and overall attempt meant to me. I doubt I'll ever write this openly again. It's art. Cheers.*

Oh, also.. We were expected to include a citation list in the allotted page count for the grant. Please allow that to excuse my minimalist citation style in this document. All of the work and main ideas are found through these few references. There is a heavy-hitting neuromechanistic idea inside this.

A. Background and Significance

Specific aims:

Chronic stress has long been known to contribute to the precipitation of a wide variety of psychological and physical disease states, but this knowledge has not resulted in

development of preventative strategies [1,2]. Caryophyllene is a relatively unknown phytocannabinoid, which has been demonstrated in a small amount of research to have promising medical properties that may broadly protect neural, hormonal, psychological, and cognitive functioning from chronic stress-induced deterioration. Importantly, caryophyllene may prove to be a valuable and accepted medical cannabinoid due to its lack of psychoactive effect, widely applicable adaptogenic (stress-buffering) properties, and status as an FDA-approved food additive. Caryophyllene interacts directly with the endogenous cannabinoid system by binding at CB2 receptors (with almost 15x greater binding affinity for CB2 receptors than cannabidiol/CBD) on neuroglial and immune cells in a manner which reduces inflammatory responses and oxidative stress in the brain and body [3-5]. Facilitation of CB2 receptor activity has been shown to protect physical, neural, cognitive, and emotional functioning in several in vivo models of stress, suggesting that CB2 receptor activation by caryophyllene will prove a valuable medical strategy for protecting and correcting a wide variety of stress-related disruptions of health [6-8]. Information about the adaptogenic capabilities of caryophyllene will introduce another therapeutic strategy to Colorado medical cannabinoid patients, but will also generalize to other CB2 receptor agonists such as CBD, and will increase the understanding of the roles of the endocannabinoid system in protecting the brain and body from stress-related damage. Our proposed research will lead to warranted advancement in medical cannabinoid philosophy and broadening of the understanding of human conditions that may benefit from cannabinoid therapies (which is currently limited in Colorado compared to conditions recognized in other states). Our research will

also directly apply to stress-induced disruptions of the brain and body (related to hyper/hypocortisolism, inflammation, and oxidative stress) which can contribute to development and/or exacerbation of all disease states currently recognized in Colorado medical marijuana philosophy. Potential distinctions in therapeutic characteristics of various phytocannabinoid CB2 receptor agonists, as well as strategic combinations of cannabinoids will be of interest in future research and will improve and refine medical cannabinoid strategies for specific conditions and patient populations. We propose to research the adaptogenic capabilities of caryophyllene using rodent models of chronic stress, which reliably produce physical, behavioral, and cognitive symptoms that are hallmarks of stress-related disorders including anxiety, depression, and post-traumatic stress disorder (PTSD) as well as a self-perpetuating stress-induced disruption of health that contributes to many disease states. A growing body of evidence demonstrates a multifaceted role of the endogenous cannabinoid system in protecting the brain and body against stress-related damage [9,10], and implicates stress-induced damage of this system as a primary factor in the etiology of various psychological disorders and diseases related to stress [5,10]. This important body of research and medical potential is not yet acknowledged by current Colorado medical marijuana legislation. We propose a novel mechanism by which CB2 receptor activation by caryophyllene can protect the endocannabinoid system and prevent stress-related damage. A main strength of this proposal is the potential ability of non-psychoactive caryophyllene to demonstrate medical benefits of cannabinoids on cognitive and psychological functioning, which may have previously been eclipsed by side effects of the psychoactivity of more traditionally

researched cannabinoids such as tetrahydrocannabinol (THC) and cannabinol (CBN). Finally, we feel that a major strength of the proposed research includes the wealth of information that will be generated from our battery of quantitative tests that will be performed in a highly controlled environment, which includes invasive neural measures that cannot be performed in human populations, but have direct relationship to human psychological conditions. This preclinical research can lead to multiple high impact scientific publications and may prove a launching point of several lines of impactful cannabinoid research with immediate medical implications. We propose the following studies:

Aim 1: Determine whether daily administration of caryophyllene can prevent chronic stress-induced development of behavioral, emotional and cognitive dysfunctions characteristic of anxiety, depression, PTSD, and general stress-related decline. Measures to include rodent models of anxious behavior, social behavior, stress hyper-reactivity, learned helplessness, anhedonia, memory acquisition and maintenance, and cognitive flexibility. The mechanistic involvement of CB2 receptor activation in these protective capacities of caryophyllene will be tested by blockade of CB2 receptors with a specific pharmacological antagonist.

Aim 2: Determine whether daily administration of caryophyllene can prevent accumulation of chronic stress-induced damage to neural tissue and brain/body systems known to contribute to stress-related dysfunction and disease states.

Measures to include biological markers of: neural endocannabinoid system activity, oxidative stress, inflammatory cytokines, neural growth hormone Brain-Derived Neurotrophic Factor (BDNF), stress-related neurotransmitter corticotropin releasing factor (CRF), and transiently expressed gene c-*fos* (a quantitative marker of cellular activity used to measure hyper-reactivity or decreased functionality of neural and endocrine tissue). The ability of CB2 receptor antagonism to prevent the protective capacity of caryophyllene on these tissue measures will be used to confirm CB2 receptor necessity in this protective therapy.

Aim 3: Determine whether repeated administration of caryophyllene can prevent stress-induced alterations of neuroendocrine (HPA axis) functioning associated with human psychological disorders and disease states. Comparisons to acute activity of caryophyllene. Measures to include stress-induced, biological markers of cellular activity in hypothalamic, pituitary and adrenal tissue (to examine possible hyperactivity, arrhythmicity, and decreased or disrupted function; e.g. c-*fos*, CRF, POMC, clock genes, endocannabinoid system markers); dexamethasone suppression of CORT (glucocorticoid negative feedback), ACTH-stimulated release of CORT (adrenal sensitivity, sufficiency), and mapping of circadian HPA axis activity (strength of rhythmicity). The contribution of central compared to peripheral CB2 receptors in the preventative capabilities of caryophyllene on these measures will be tested using direct microinjection into the cerebral ventricular system. Lack of CB1 receptor-dependent

modulation of activity will also be tested with acute stress after caryophyllene pre-treatment.

Significance

Patterns of purposeful human marijuana consumption have been useful in directing researchers towards the specific disease states in which cannabinoids are proving to have medical benefits. The physical mechanisms underlying these medical benefits directly relate to interaction of phytocannabinoids with the endogenous cannabinoid system via actions at CB1 and CB2 receptors. As such, scientific information about the role of endocannabinoid activity in the functioning of normal, healthy organisms, as well as identification of the human disease states in which endocannabinoid signaling is deficient is laying an important groundwork for the multifaceted medical potential of cannabinoids, and has been the recent focus of a collective research movement. Very quietly, an impressive body of academic research is coming together in support of medical use of marijuana and/or specific cannabinoids in the prevention and treatment of disease states known to relate to chronic stress. This research is illustrating a role of endocannabinoid signaling as a many-sided protective mechanism of the brain and body, and is implicating disruption of this protective mechanism as a main causative factor in human vulnerability to disease and dysfunction. In this application, we will present both of these sides of the recent research and offer a novel medical cannabinoid strategy. In line with the collective intentions and interests of the Colorado medical marijuana research program and the population represented by this collective

effort, our proposed research would importantly advance the knowledge of the therapeutic potential of cannabinoids in several ways. These advancements include: 1) Introduction of a relatively unknown cannabinoid (caryophyllene) into the medical marijuana pharmacopoeia, 2) Examination of a novel and widely applicable strategy of combating stress-induced deterioration of health, 3) Laying of groundwork for the much needed expansion of the limited list of disease states eligible for medical marijuana treatment in Colorado, and 4) A furthering of knowledge about the endogenous cannabinoid system which will be useful in future refinement and sophistication of cannabinoid-based medical strategy.

An additional, multi-layered benefit of our proposed course of research may not be immediately obvious, but relates to the strategic use of a phytocannabinoid that enjoys a unique federal legal status as an FDA approved food additive. This negates a need for federal licensing and supervision of our course of study, and will importantly allow for unimpeded creative scientific freedom in exploring medical applications of this cannabinoid. This work will be promptly completed in an academic laboratory with a strong history of federally funded stress research, scientific publications in top tier journals, and presentation at prominent academic research conferences. Completion of the work in our proposal and subsequent influence of the findings in medical, scientific, and political circles will not be subjected to the potential blockade or alteration foreseeable for proposed research programs that have to yet to secure federal approval and schedule 1 licensing. The robust medical actions of caryophyllene demonstrated in

the slowly growing number of scientific publications suggest that the federal designation should not be taken to imply a lack of medical potency. In the Colorado medical marijuana scene, caryophyllene-rich strains are starting to draw the attention of patients in a grassroots manner that appears to be in part influenced by the suggested potential of research literature but likely demonstrates a desirable profile of medical characteristics unique to this sesquiterpene phytocannabinoid. Given that the rise in grassroots popularity of cannabidiol (CBD) intelligently preceded the scientific validation of medical potential that is now unfolding, CO mmj patient appreciation of caryophyllene appears to support a modest investment of patient-generated funds in scientific examination of this compound. As with the more popular CBD, caryophyllene is known to lack psychoactivity due to absence of action at CB1 receptors while exerting broad medical effects through direct binding at CB2 receptors [3]. This distinction has proven desirable in patient populations, and has contributed to a rise in medical marijuana approval that extends far beyond our own state (and promises to ultimately benefit a larger patient population). This specificity in action (at CB2 receptors) can circumvent side effects related not only to the psychoactive properties of CB1 receptor action in the brain, but to potential side effects of CB1 receptor overstimulation in peripheral tissues such as adipose and adrenal tissue.

Our proposal stems from research that suggests chronic stress results in a self-perpetuating state of endocannabinoid deficiency affecting responsibilities of both CB1 and CB2 receptor activity in the brain and body. Recent scientific advancement in the

understanding of the widespread functions of the endocannabinoid system support the idea that a disruption of these functions contributes to a general susceptibility to stress and a variety of symptom patterns and disease states. Collectively, this research suggests that the common human pattern of regular, low dose marijuana use can protect one's health from disruption due to stress-related issues and prevent the initiation of multiple disease states. Our proposed research would help to confirm this possibility, which has not been rigorously tested. However, the growing mmj patient demand for non-psychoactive cannabinoid medications, and the potential experience of side effects related to CB1 receptor stimulation support a need to ultimately refine this strategy. We hypothesize that a pharmacological increase in CB2 receptor activity will emphasize beneficial effects of this strategy and importantly protect signaling at CB1 receptors, while bypassing side effects relating to CB1 receptor overstimulation. Our proposal is based on a novel idea that regular CB2 receptor stimulation can be a backdoor mechanism to protect the entire endocannabinoid system from stress-induced damage. The information generated by our proposed studies would importantly generalize to other CB2 receptor agonists (e.g. CBD), but would introduce another pharmacological cannabinoid strategy to this young medical field. Caryophyllene may prove a more appropriate medication for some patient populations and medical conditions but also may be important in combination cannabinoid strategies. Our proposal is based on a large body of recent research that has not been taken into account by the current CO medical marijuana legislation and philosophy, and we feel this one-time funding event affords an exciting opportunity to introduce and develop it.

Chronic stress as a main factor in the etiology of psychological disorders and disease states: Chronic stress is known to contribute to the initiation and exacerbation of a wide variety of psychological and physical disorders, as well as a general decrease in whole organism health that includes disruption of physical, cognitive, and emotional functioning. Stress-related psychological disorders such as anxiety, depression, and post-traumatic stress disorder (PTSD) present substantial individual and societal burden. Treatment of these disorders with current pharmacological therapies is often incomplete, and introduces significant side effects that can further disrupt the health and functioning of the individual. Development of improved strategies to treat these disorders is a present concern, and should take into account the role of chronic stress in the etiology and maintenance of these states. The damaging influence of chronic stress is multi-faceted and interactive, and includes: 1) Psychological and emotional disturbance, 2) Disruptions related to overstimulation of the hypothalamic-pituitary-adrenal (HPA) axis and damaging influence of excessive cortisol elevation and disrupted adrenal sensitivity, 3) Altered circadian rhythm and the resulting suboptimal functioning of many systems in the body, 4) Accumulation of widespread cellular damage from increased oxidative stress, 5) Damaging immune and glial cell-mediated hyper-inflammatory states in the body and brain, 6) Disruption of cognitive functions such as memory and cognitive flexibility, and 7) disrupted reward processing. Neural and endocrine structures that are disrupted by chronic stress in a way to contribute to symptom patterns of anxiety, depression, and PTSD include: the amygdala

(emotionality, stress-reactivity), the hippocampus (memory, stress-reactivity), the medial prefrontal cortex (mPFC, cognitive flexibility, stress-reactivity, learned helplessness), and the HPA axis (hyper/hypocortisolism, circadian rhythm). Our proposal stems from an understanding that many of these stress-related disturbances are not only consequences of chronic stress, but are also causative of further disruption, leading to an interactive state of decreased health that is often self-perpetuating. Recent research places the endogenous cannabinoid system in a position to prevent or reduce many aspects of this pattern of stress-related disruption of health, suggesting an ability of caryophyllene to offer widespread protection from stress-induced dysfunction.[10-19]

The endogenous cannabinoid system protects against stress-induced dysfunction: The endogenous cannabinoid system includes CB1 and CB2 receptors and the ligands that indiscriminately bind and activate these receptors (lipids anandamide and 2AG), as well as enzymes responsible for synthesizing (DAGL) and degrading (MAGL, FAAH) these ligands. The endocanabinoid (eCB) system is widely expressed in the brain and body and exerts mild inhibitory regulation of cellular activity in neural, endocrine, and immune systems. Marijuana interacts with this system through phytocannabinoids (e.g. THC, CBN, CBD, caryophyllene), which bind at CB1 and/or CB2 receptors due to structural similarity to endocannabinoids (eCBs). eCB activation of CB1 receptors on neurons in the amygdala and extended amygdala afford inhibitory dampening of psychological reactivity to stressors and resulting anxiety. The eCB system indirectly regulates HPA axis responses to psychological stress through this

inhibitory action in the amygdala but may directly dampen stress-induced HPA axis responses and resulting cortisol levels through activation of inhibitory CB1 receptors at all three structures intrinsic to the HPA axis. eCB activity at CB1 receptors in the mPFC and hippocampus helps to dampen stress-induced stimulation of these two structures by cortisol and excitatory monoamine responses to psychological stress. Disruption of eCB/CB1 activity in the mPFC and hippocampus can exacerbate the well-known structural and functional damage that results from chronic stress (mPFC: “hypofrontality”, decreased BDNF levels and cognitive flexibility; hippocampus: decreased neurogenesis, BDNF levels, and memory capacity). THC exerts its psychoactivity by binding at CB1 receptors throughout the brain (relaxation at low doses, increasing intoxication and sedation at higher doses) in a way to mildly inhibit neuronal activity. Though THC could be useful in reducing stress-reactivity in the amygdala and HPA axis, excessive use can decrease activity in the hippocampus and mPFC, leading to the common temporary side effects of decreased memory and attention functioning, and may disrupt adrenal functioning important to circadian rhythm. CB2 receptors are predominantly located on immune cells in the body, and microglial cells in the brain, through which the eCB system dampens inflammatory responses and the resulting oxidative stress. This mechanism is protective in situations of chronic stress-induced hyper-inflammatory states. Excitatory properties of inflammatory cytokines in neural and HPA axis activity are thought to contribute to neural and endocrine disruptions characteristic of anxiety, depression, and PTSD, and are protectively dampened by CB1-mediated inhibition via eCBs (which are widely released

in response to the inflammatory state and resulting neural excitation). Widespread depletion of eCB levels or decreased receptor functioning (CB2 and/or CB1) would result in worsening of hyper-inflammatory states and their related damages, and may contribute to their initiation.

The locations of inhibitory CB1 and CB2 receptors allow the eCB system the ability to combat: 1) Increased psychological stress-reactivity (CB1 receptor-mediated), 2) Hypercortisolism (CB1 receptor-mediated), and 3) Hyper-inflammation (CB2 receptor-mediated) resulting from chronic stress. Our proposal is based on the idea that disrupted psychological, HPA axis-related, and inflammatory responses in chronic stress interstimulate to form a self-perpetuating state of dysfunction, and that this state can be prevented (and likely corrected) by targeting any one of the three disruptions. We predict that a CB2 receptor agonist (caryophyllene) would capitalize on this interrelationship in a way to protect CB1-mediated regulation of psychological and neuroendocrine activity, while avoiding the side effects and psychoactivity related to CB1 receptor activation. [5,9,10,20,21]

Disruption of the endocannabinoid system by chronic stress: An important aspect of our proposal relates to eventual disruption of the eCB system by chronic stress, which is supported by several lines of evidence. Rodent models of chronic stress have been reported to result in reduced measures of multiple aspects of eCB activity, including: CB1 receptor expression, binding, functioning, and mRNA; CB2 receptor mRNA; and tissue levels of eCB anandamide in various structures of the brain, including

mPFC, amygdala, hypothalamus, and hippocampus. These disruptions of the eCB system can also result from chronic glucocorticoid elevation/administration. [10]

eCB system deficiency in human conditions related to stress: Circulating eCB levels have been measured to be **decreased** in human disorders related to chronic stress, including: Non-medicated female major depressive disorder (anandamide and 2AG), anxiety (anandamide) and PTSD (anandamide and 2AG). A post-mortem study of PTSD patients revealed decreased CB1 receptors in the frontal cortex (anterior cingulate), and a PET-imaging study has measured decreased CB1 receptor activity in the brains of PTSD patients, that significantly correlated with symptom prevalence and severity. [10]

Caryophyllene as a strategy to reduce stress-induced disruption of health by protecting the endogenous cannabinoid system: Caryophyllene (E-β-caryophyllene) is the most substantial sesquiterpene in cannabis (measuring as high as 35% in cannabis essential oil) and is most concentrated in the resin glands of female plants. Caryophyllene has recently been recognized as a cannabinoid that binds selectively at CB2 receptors. Comparison of reported binding affinities suggests that caryophyllene has almost 15x greater binding affinity for the CB2 receptor than fellow phytocannabinoid CBD. Analyses of marijuana indicate that caryophyllene is substantially present in all species of cannabis (sativa, indica, ruderalis, and hybrids). As selective breeding of marijuana plants has recently been used to drastically increase

CBD levels and decrease THC levels (such as the “Charlotte’s Web” strain with a reported 17% CBD and 0.5% THC), it appears possible to develop strains with relatively high levels of caryophyllene. Interestingly, caryophyllene is also a constituent of other plant essential oils such as oregano, hops, and black pepper. The possibility of extracting caryophyllene from multiple plant sources suggests that caryophyllene-based medications should maintain affordability and availability. Treatment with caryophyllene in models of stress has been shown to substantially decrease inflammatory responses and oxidative stress, but it is untested whether caryophyllene can mediate protection of the many behavioral, psychological, and physical functions that are predictably damaged by chronic stress. Evidence to support the ability of caryophyllene to offer more widespread protection comes from research on CB2 receptor facilitation, and direct association of inflammation and oxidative stress with eventual widespread disruption of functioning in the measures of interest. Caryophyllene has been demonstrated to have potent anti-inflammatory and anti-oxidative capacities via oral administration, which will likely prove the most acceptable route. [2-5,13,20,22]

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