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Neuroendocrine and Behavioural Alterations Elicited by Chronic Unpredictable Stressor Challenges in Stressor-Susceptible and Resilient Mouse Strains

A thesis submitted to the Department of Psychology and the Faculty of Graduate Studies and Research of Carleton University in partial fulfillment of the requirement for the degree of

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Abstract

Stressors profoundly influence hypothalamic-pituitary-adrenal (HPA) functioning; however, marked differences exist between strains of mice that vary in their reactivity to stressors. In particular, in response to a variety of stressors BALB/cByJ mice exhibit marked behavioral signs of anxiety as well as heightened neuroendocrine and neurochemical responses relative to stressor-resilient C57BL/6ByJ mice. In the present investigation, the influence of acute and chronic sustained stressors (mild/moderate insults applied twice daily over 7 weeks,) was determined in these mouse strains. The latter stressor was hypothesized to provoke excessive demands on neuronal functioning (allostatic overload), thus favoring vulnerability to stressor-related illnesses such as depression and anxiety, particularly within the stressor-reactive BALB/cByJ mice. Quantitative immunohistochemistry, in combination with a deconvolution procedure to acquire high-resolution confocal images from widefield fluorescence microscope images, was used to assess the individual and co-expression of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), as these peptides synergistically stimulate glucocorticoid release. The peptides in the ME were, however, unaffected by the Strain or the Stressor condition. In contrast, the BALB/cByJ and C57BL/6ByJ mice displayed opposing outcomes in response to the acute stressor with respect to both the levels of CRH receptors and CRH peptide in the PFC and CeA, respectively. The position is offered that the CRH changes in these strains may underlie their relative vulnerability to anxiety elicited by stressors, and thus, may be relevant to analyses of anxiety processes in humans.

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Table of contents

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List of Figures

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List of abbreviations

ACTH – adrenocorticotropic hormone

AI – agranular insular cortex

ANOVA – analysis of variance

AVP – arginine vasopressin

AVP1b – AVP receptor subtype 1b

BLA – basolateral nucleus of the amygdala

BNST – bed nucleus of the stria terminalis

CBF – cerebral blood flow

CeA – central nucleus of the amygdala

CORT – corticosterone

CRH – corticotropin-releasing hormone

CRH_1 – CRH receptor subtype 1

CRH_2 – CRH receptor subtype 2

GC – glucocorticoid

hnRNA – heteronuclear ribonucleic acid

HPA – hypothalamic-pituitary-adrenal

ip – intraperitoneal

ir – immunoreactivity

LPS – lipopolysaccaride

LO – lateral orbital cortex

MC – mineralocorticoid

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ME – median eminence

MO – medial orbital cortex

mPFC – medial division of the PFC

mRNA – messenger ribonucleic acid

NA – numerical aperture

NE – norepinephrine

oPFC – orbital division of the PFC

PBS – phosphate-buffered saline

PFC – prefrontal cortex

POMC – pro-opiomelanocortin

PSF – point spread function

PVN – paraventricular nucleus of the hypothalamus

r – receptor

ROI – region of interest

SEM – standard error of the mean

5-HT – 5-hydroxytryptamine (serotonin)

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Introduction

Stressful events, together with a constellation of other experiential and organismic variables, contribute to the provocation and exacerbation of depressive illness. Indeed, in humans, stressful events frequently precede the onset of depression, and may also worsen this condition in those with a history of depression (Arborelius et al., 1999; Hammen et al., 1992). The stressors that promote depressive illness are not limited to traumatic events, but may include the cumulative actions of less severe stressors (Anisman et al., 2002). In this regard, stressors that occur unpredictably on a protracted basis may result in certain biological systems being overly taxed (i.e. allostatic overload), hence favoring pathological outcomes (McEwen, 2000).

Paralleling the effects reported in humans, among infrahuman animals, stressors may elicit behavioural disturbances (e.g. anhedonia) that are reminiscent of the symptoms that characterize depression (Anisman et al., 2002; Weiss et al., 1981; Willner, 1997). Based on analyses of the biological and behavioural changes that are evoked by stressors, it was suggested that alterations of neurochemical, neuroendocrine and immunological systems contribute to the development of illness. Furthermore, it was argued that the individual behavioural differences that exist in response to stressors might be attributable to the diverse neurochemical changes exerted by such treatments. In this respect, genetic and experiential factors, such as early life events (including maternal care) may promote either increased resistance or vulnerability to stressors encountered later in life (Anisman et al., 1998a; Liu et al., 1997; Meaney, 2001).

Although it was long thought that serotonin (5-HT) variations are fundamental in the provocation of depression, increasing evidence has pointed to corticotropin-releasing

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hormone (CRH) playing a fundamental role in the evocation of depressive symptoms. In this respect, elevated levels of CRH have been reported in patients with major depression (Nemeroff, 1984). This includes CRH within the hypothalamus (Raadsheer et al., 1994), as well as variations of this peptide at extrahypothalamic regions, such as the prefrontal cortex (PFC) and amygdala (Nemeroff, 1988; Schulkin et al., 1998). In the present investigation, we evaluated the impact of a chronic, unpredictable stressor regimen on aspects of hypothalamic-pituitary-adrenal (HPA) functioning, a system that is thought to be integral in the stress response. Specifically, the changes of expression of two neuroendocrine peptides, CRH and arginine vasopressin (AVP), were assessed in various stressor-sensitive regions, as these have been thought to synergistically stimulate adrenocorticotropic hormone (ACTH) and hence corticoid release, thus contributing to the protracted effects of chronic stressors.

In order to model the individual differences in stressor susceptibility that are seen among humans, these changes were assessed in two strains of mice, one of which displays high levels of anxiety and vulnerability to stressor-elicited HPA changes (BALB/cByJ), and the other, a more hardy, resilient mouse strain (C57BL/6ByJ) that ordinarily exhibits low levels of stressor-elicited behavioural reactivity. It was hypothesized that in mice of the BALB/cByJ strain the chronic stressor regimen would lead to more pronounced variations of CRH within the PFC and amygdala, as well as CRH and AVP within the median eminence (ME), favoring behavioural signs of depression and anxiety.

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The hypothalamic-pituitary-adrenal axis

The major central component of the HPA axis is the paraventricular nucleus of the hypothalamus (PVN). Populations of neurosecretory cells in the dorsal medial parvocellular division of the PVN produce the ACTH secretagogues, CRH and AVP, respectively (Herman & Cullinan, 1997). The majority of these neurons only synthesize CRH, while some produce both CRH and AVP (Whitnall, 1993). Projections to the external zone of the median eminence extend to storage vesicles, where CRH and AVP are secreted into the hypophyseal portal system. CRH and AVP are then transported to the anterior pituitary via a capillary network of long portal blood vessels, where they act synergistically to increase the expression of the ACTH precursor pro-opiomelanocortin (POMC), and ultimately stimulate ACTH release (Antoni, 1986). The secreted ACTH reaches the adrenal cortex through the bloodstream, where it stimulates the secretion of glucocorticoids (GCs) (namely, cortisol in humans, and corticosterone in rodents), which are the final effectors of the HPA axis (Chrousos, 1998).

Circulating GCs exert extensive physiological effects via widely distributed GC receptors that are situated throughout the brain and body. Physiological and psychological disturbances of any kind reliably provoke changes in plasma levels of GCs, such that it is widely used as a marker to measure the stress response in human and animal studies. These changes are generally of adaptive value, as they help mobilize energy stores, improve cardiovascular tone, stimulate inflammation, and other changes in immune functioning that are beneficial to the organism, as well as affecting neural and behavioural responses that help them cope with the stressor (Sapolsky et al., 2000; Whitnall, 1993). Moreover, activated GC receptors along with GC responsive elements,

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regulate the transcription of genes that are involved in various stress response mechanisms (Chrousos, 1998).

Among the various effects of GCs, one of the most important may be the inhibitory feedback that they provide on the HPA axis, limiting its prolonged activation. Major inhibitory control is provided by circulating GCs binding directly to receptors in the PVN as well as the hippocampus, ultimately limiting the release of CRH, which in turn inhibits the release of ACTH and further release of GCs. Evidence of this is the finding that injection of GCs into the PVN down-regulate CRH mRNA (messenger ribonucleic acid), and inhibit medial parvocellular neurons (Whitnall, 1993). Similarly, GC receptors in the anterior pituitary allow for direct inhibitory feedback on ACTH release.

Role of arginine vasopressin on HPA activity

Typically, AVP is recognized for its role in osmotic regulation, being secreted in response to dehydration and hypotension (Whitnall, 1993). In this context, AVP originates from magnocellular neurons of the PVN and supraoptic nucleus whose axons terminate within the posterior lobe of the pituitary, where it is transported to and released into the peripheral circulation (Aguilera & Rabadan-Diehl, 2000). Arginine vasopressin of parvocellular origin however, has potent stimulatory effects on adrenocorticotropic secretion (Aguilera, 1994). In contrast to CRH, it seems that AVP is a weak stimulator of pituitary ACTH secretion. However, AVP was found to potentiate the effect of CRH on ACTH secretion, particularly when being co-released with CRH at the external zone of the ME (Abou-Samra et al., 1987; Gillies et al., 1982; Rivier and Vale, 1983).

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Limbic contributions to HPA regulation

Regulation of HPA functioning is not limited to GC negative feedback, but also includes indirect pathways between the HPA axis and various interconnected brain regions. It is suggested that these regions are required for the maintenance of basal HPA functioning (Herman & Cullinan, 1997). Among these are the hippocampus, prefrontal cortex (PFC) and amygdala, of which the hippocampus has been the most widely studied, likely owing to its high density of GC and mineralocorticoid (MC) receptors (Herman & Cullinan, 1997). In fact, hippocampal damage has been shown to potentiate the secretion of GCs, through an increase in parvocellular CRH and AVP (Herman et al., 1995; Sapolsky et al., 1991), indicating an inhibitory role for the hippocampus in HPA functioning.

The PFC has also been shown to widely express GC receptors (Ostrander et al., 2003; Patel et al., 2000), thus allowing for inhibitory feedback control over areas of the HPA axis. Lesion studies have revealed that damage to the medial division of the PFC (mPFC), as in the case of the hippocampus, significantly increases ACTH and GC responses to stimuli (e.g., stressors) that ordinarily stimulate HPA activity (Diorio et al., 1993). While the mPFC does not directly innervate the PVN, many indirect connections via brainstem nuclei, the bed nucleus of the stria terminalis (BNST) and other hypothalamic regions allow for control over HPA functioning (Herman et al., 2003). Additionally, the orbital division of the PFC (oPFC) has been reported to have connections with the amygdala as well as the hypothalamus (Drevets, 2000), regions known to be involved in mediating HPA activity. However, it is unknown whether the

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orbital division of the PFC exerts inhibitory control over HPA activity, as in the case of the mPFC.

The various nuclei contained within the amygdala have numerous inter-connections to regions involved in the stress response, including direct projections to the PVN (as well as indirect projections to the PVN via the BNST), supporting its role in moderating HPA activity (Herman et al., 2003). Lesions to the central nucleus of the amygdala (CeA), in particular, have been shown to cause depletion of CRH from the ME (Beaulieu et al., 1989). Moreover, the expression of CRH in this region further supports its involvement in HPA regulation (Pett et al., 2000).

Stressors and HPA functioning

Stressors induce a wide array of neurochemical, neuroendocrine and immunological (including cytokine) changes. In evaluating the behavioural effects of stressor events, particular attention has been devoted to the impact of challenges on monoamine as well as on certain peptidergic (primarily CRH and AVP) systems.

The data concerning CRH changes associated with stressors have been extensively examined, and there is reason to suppose that this peptide is also affected by stressor chronicity. To a considerable extent, the focus of this research has involved CRH changes associated with HPA functioning, and a relatively smaller number of studies focused on chronic stressor effects on CRH within other brain regions. The sections that follow provide an overview of some of the known effects of acute and chronic stressors on HPA functioning, with particular attention to CRH variations within stressor sensitive regions including the PFC, ME, and amygdala.

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Neurochemical and neuroendocrine effects of acute and chronic stressors

As indicated earlier, acute stressors promote the release of various neurotransmitters and peptides, followed by normalization to pre-stress levels through intrinsic regulatory mechanisms once the stressor has terminated (Sapolsky et al., 2000). These responses allow the organism to appropriately deal with the stressor. However, when the stressor is sufficiently protracted, those neurochemical changes that ordinarily act to facilitate well being, may culminate in adverse reactions on various organ systems, and may favor the development of behavioural pathologies.

In the case of central neurochemical changes, acute stressors generally increase amine utilization, and if the stressor is sufficiently severe, then the release of monoamines may exceed their synthesis, resulting in a net reduction of transmitter levels (Tannenbaum & Anisman, 2003; Weiss et al., 1981; Shanks et al., 1994; Irwin et al, 1986). Chronic stressors, interestingly, may instigate a compensatory increase of monoamine synthesis, leading to an elevation of levels (Anisman et al., 1987; Irwin et al, 1986; Adell et al, 1988; Nisenbaum et al., 1991). This is especially true when a particular stressor is applied on a predictable basis, and may explain the adaptation that is typically seen with this type of stressor regimen. In contrast, when chronic stressors are varied, and are applied on an unpredictable basis, then these compensatory increases are less pronounced or absent, favoring the development of depressive states (Willner, 1997; Tannenbaum et al., 2002).

With regard to HPA activity, acute stressors stimulate the release of PVN peptides (CRH and AVP), ultimately promoting the secretion of pituitary ACTH and adrenal GCs as previously described. Levels of these peptides are kept in check by inhibitory feedback

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mechanisms. Chronic stressors on the contrary, induce a dysregulation of HPA activity, possibly owing to wear and tear on the system exacted by repeated activation (McEwen, 2000). The increase of GC secretion induced by chronic stressors has been shown to be insufficient to block the up-regulation of CRH and AVP mRNA expression in the parvocellular PVN (Herman et al., 1995). These data suggest that feedback inhibition is disrupted, producing exaggerated GC responses to acute stressors encountered later on. These effects are long lasting, as they can be elicited weeks, even months after the last stressor exposure (Tilders et al., 1999; Dallman et al., 2004). This may also, in part, be due to a down-regulation of GCs receptors in major regulatory regions, such as the hippocampus (Herman et al., 1995).

In addition to disturbed inhibitory feedback mechanisms, chronic stressors induce long lasting phenotypic changes within CRH cells that terminate in the ME. These changes presumably allow the effects of chronic stressors to be sustained. Following acute stressors, significant increases in the expression of hypothalamic CRH have been reported (Cook, 2004; Imaki et al., 2001; Pinnock & Herbert, 2001; Makino et al., 1999; Makino et al., 1995). However, in situations when HPA activation is protracted, there is a gradual increase in the number of AVP-positive neurons in the external zone of the ME, as well as a shift in the CRH/AVP signal from CRH being the major stimulator for ACTH secretion, to both AVP and CRH as stimulators of ACTH secretion (De Goeij et al., 1992; Kiss and Aguilera, 1993). Similar to chronic stressors, adrenalectomy in rats significantly increased AVP staining within parvocellular CRH-immunoreactive neurons (De Goeij et al., 1993; Dallman, 1993). These data suggest that increased AVP co-localization is indicative of hyperactivity of neurons at this level of the HPA axis.

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Effects of acute and chronic stressors on neuropeptide variations within limbic regions

Stressors profoundly influence activity in the various regulatory limbic regions of the HPA axis. Presumably, these higher-order brain regions process many of the cognitive, emotional and behavioural changes typically elicited by stressors. Moreover, anticipatory stress responses (i.e. those that are elicited in the absence of a physiological challenge, generated by a conditioned stimulus or innate predispositions) are integrated within these regions (Herman et al., 2003)

Acute stressors have been shown to activate the immediate-early gene, c-fos, within the medial and orbital divisions of the PFC (Ostrander et al., 2003; Yokoyama & Sasaki, 1999; Handa et al., 1993). While this holds true for stressors that involve higher-order emotional processing (i.e. processive stressors such as restraint, novel environment and loud noise), systemic stressors such as an osmotic challenge, lipopolysaccharide (LPS) injection, ether exposure and hypoxia do not elicit robust activation within the PFC (Figueiredo et al., 2003; Yokoyama & Sasaki, 1999; Herman & Cullinan, 1997; Handa et al., 1993). These data indicate a regulatory role for the PFC over HPA activity, however, it appears that this role may be stressor-specific. Indeed, the inhibitory effects of the mPFC over HPA activity have been shown to apply only to processive stressors (Figueiredo et al., 2003; Herman & Cullinan, 1997).

Chronic stressors have been shown to produce a down-regulation of GC receptors in the mPFC inducing widespread behavioural and cognitive disturbances (Mizoguchi et al., 2004). Additionally, chronic stressors were shown to provoke atrophy of dendritic branches in the mPFC, which may contribute to cognitive deficits associated with this treatment (Cook and Wellman, 2004; Radley et al., 2004). These changes may stem from

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a reduction of the responsiveness of neurons in this region to GCs, possibly through receptor down-regulation (Mizoguchi et al., 2004). While the involvement of the mPFC in the effects of chronic stressors has been consistently reported, limited information is available regarding the role of the oPFC in response to chronic stressors. Yet, chronic social stress was associated with long-term reductions of serotonergic activity within the oPFC (Fontenot et al., 1995). Moreover, metabolic changes within this region have been implicated in depression and anxiety (Drevets, 2000; Grachev & Apkarian, 2000). It is not known whether the oPFC exerts inhibitory regulation over HPA activity (as in the case of the mPFC), however, it is possible that the two regions play parallel roles in HPA functioning.

To date, limited data are available concerning changes of CRH peptide and CRH receptor expression within areas of the PFC in responses to acute or chronic stressors, despite the fact that they have been implicated in depression (Merali et al., 2004). As such, it is conceivable that the PFC modulates the depressive-like symptoms induced by chronic stressors.

Acute stressors have repeatedly been shown to stimulate CRH levels in the amygdala (Kalin et al., 1994; Hand et al., 2002; Merali et al., 2003). This is of particular significance as the amygdala is thought to be involved in determining the emotional significance and emotional appraisal of external and internal stressors (Tafet et al., 2003), and may influence the acquisition and expression of fear and anxiety (Davis, 1998). In this respect, stimulation of CRH within the amygdala, particularly the CeA, may be essential in promoting anxiety responses (Dunn and Berridge, 1990). It was shown that predator exposure increased CRH in both the amygdala and PVN, and a CRH antagonist

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applied to the amygdala immediately prior to the stressor, significantly reduced anxiety responses to the same stressor applied 2 days later (Cook, 2002). Furthermore, a psychosocial stressor increased CRH mRNA levels in the CeA (Makino et al., 1999). While acute stressors such as predator exposure and novel environment reliably increased CRH in the CeA, the effects of attenuating the anxiety induced by these stressors by CRH antagonists has been inconsistent, suggesting that amygdaloid CRH release may not be necessary for the expression of anxiety responses (Merali et al., 2004b). It seems likely that although CRH release within this region is involved in some aspect of the stress response, the function of these CRH variations may be stressor-specific (Merali et al., 2004b). Moreover, it is supposed that these stressors may stimulate distinct pre-wired CRH-independent neural circuits that may be involved in fear and anxiety-like behaviours.

There are definite effects of acute stressors within the amygdala, but limited data are available concerning the effects of chronic unpredictable stressors within this region, especially the CeA. It was shown that chronic immobilization induced dendritic remodeling within the amygdala, which is thought to subserve the enhanced anxiety-like behaviour seen even days after the last stressor exposure (Vyas et al., 2004). Additionally, chronic psychosocial stressors resulted in an up-regulation of CRH receptors in the lateral amygdala and CeA (Fuchs and Fugge, 1995), and increased CRH mRNA was also evident under such conditions (Albeck et al., 1997). Although a role for CRH in the CeA in contributing to pathology (e.g., anxiety, depression) associated with chronic stressors cannot be precluded, it is still premature to postulate the limiting factors for such an outcome.

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Individual differences of stressor reactivity

Marked individual differences exist with respect to neurophysiological stress responses that cannot be accounted for simply by the nature of the stressor. Instead, it has been suggested that organismic (e.g. age, gender, species) as well as experiential (e.g. previous stressor exposure) variables may influence neurochemical vulnerability, essentially programming biological reactivity to stressors (Anisman & Merali, 1999; Plotsky & Meaney, 1993). In this respect, early life events such as maternal care and environmental insults may drastically impact the responses to adult stressor reactivity.

To understand the processes associated with the stress response, work in our laboratory evaluated two genetically distinct inbred strains of mice, BALB/cByJ and C57BL/6ByJ, differing considerably in their physiological reactivity to stressors. Specifically, it was found that BALB/cByJ mice displayed marked neuroendocrine (i.e. CORT and ACTH) responses to stressors such as open-field exploration, restraint, footshock, forced-swim, acoustic startle stimuli, and cytokine challenge relative to the hardier C57BL/6ByJ strain (Anisman et al., 2001; Lu et al, 1998). Furthermore, BALB/cByJ mice exhibited significantly more behavioural signs of anxiety relative to C57BL/6ByJ mice in tests such as open-field emergence, elevated-plus maze, light-dark box and step-down exploration (Anisman et al., 2001). Observations in this regard indicated that some of the behavioural signs of anxiety in BALB/cByJ mice could be attenuated, at least to some degree, by cross-fostering, wherein BALB/cByJ pups were raised by a C57BL/6ByJ dam (Caldji et al., 2004; Zaharia et al., 1996). This was attributed to early life maternal care factors, as BALB/cByJ mice tend to be poorer

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mothers than C57BL/6ByJ mice, engaging in diminished licking/grooming and nursing quality of pups (Anisman et al., 1998a). In light of these findings, it was suggested that the BALB/cByJ and C57BL/6ByJ strains might be useful to assess the contribution of genetic factors and early life experiences, in determining the adult response to stressors.

Little information is available regarding the effects of chronic stressors across individuals or rodent strains that are differentially reactive to stressors. It was, however, observed that following a chronic stressor regimen, BALB/cByJ mice displayed increased behavioural symptoms of anxiety and depression relative to C57BL/6ByJ mice (Tannenbaum & Anisman, 2003). This was presumed to be due, in part, to sustained increases of amine utilization, as acute stressors provoked more pronounced changes of norepinephrine (NE) and 5-HT utilization in BALB/cByJ mice. Additionally, it was found that these strains differed with respect to basal CRH levels within the amygdala and PVN, indicating that differences of stressor reactivity in these strains are not limited to ACTH and CORT (Anisman et al., 1998b). Moreover, chronic stressors were shown to induce significantly different GC responses within these strains, and it is suggested that this may be due to differing profiles of CRH and AVP (Anisman et al., 1998b). Presumably, differences of CRH and AVP expression are what render these strains differentially reactive to stressors. As such, it is hypothesized that a chronic, intermittent stressor regimen, imposed on differing genetic backgrounds, will reveal changes of CRH and AVP expression that may partially explain differential reactivity to stressors. Specifically, it is expected that the chronic stressor regimen will elicit an increase of co-localization of CRH and AVP within the ME, as well as CRH variations within the CeA and PFC, and that these changes will be more pronounced in the BALB/cByJ strain.

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Methods

Animals

Male BALB/cByJ and C57BL/6ByJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine) at approximately 7 weeks of age, and were allowed 2 weeks to acclimatize to the laboratory setting. Throughout the study, mice were singly housed in standard polypropylene cages (27 x 21 x 14 cm). All animals were kept on a 12 hr light-dark cycle (lights on: 0800-2000 hr) in a temperature-controlled (21°C) vivarium, and were given *ad libitum* access to pellet mouse chow (5075 Ralston Purina) and tap water. Standard cage changing for all animals took place twice per week (Tuesdays and Fridays). Chronically stressed mice were housed in a separate room in the vivarium and were removed from the room during stressor sessions and behavioural testing. Animals in the control and acute stressor groups remained in the same room throughout the study, and were undisturbed until the final experimental day. All experiments complied with the current guidelines set by the Canadian Council on Animal Care and were approved by the Carleton University Animal Care Committee.

Experimental procedures: Acute and Chronic stressor regimen

At approximately 9 weeks of age, mice were randomly assigned to one of three treatments: a chronic stressor regimen, a single acute stressor, or no treatment (n = 6 per group for each strain). The chronic stressor regimen consisted of 10 different types of stressors, most of which were administered twice daily (with the same stressor applied twice on any given day) over a period of 7 weeks, with each stressor being presented on a

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pre-selected, random, unpredictable basis. On each day, the morning stressor session was completed between 8:00 AM and 11:00 AM, and the afternoon stressor session between 12:00 PM and 3:00 PM. Mice were returned to the vivarium between stressor sessions. Animals were stressed daily from Sunday to Friday, with no stressor administered on Saturdays. The acutely stressed animals only received a single stressor session, applied on the same day that chronically stressed animals received the final stressor. All animals of these two groups received the same stressor on this day (30 minutes of acoustic startle, as described later). Control animals were not exposed to any stressors, and remained in their home cages until they were ready to be euthanized for brain processing.

The chronic stressor regimen included the following stressors:

1) **restraint** in semicircular Plexiglas tubes (4.0 cm diameter x 12.0 cm long), with tails taped outside the tube to prevent them from turning while in the tube for 15 minutes;

2) **forced swim** in a plastic cylinder (30.0 cm in diameter and 27.0 cm high) filled with 20°C water for 3 minutes;

3) **open-field exploration** in a large square opaque field 80 x 80 x 60 cm, wherein mice were allowed to explore freely for 5 minutes;

4) exploration in an **elevated plus maze**, where mice were place in center portion and allowed to explore freely for 5 minutes;

5) exploration in a **Y-maze**, where mice were placed in center portion and allowed to explore freely for 5 minutes;

6) **predator fox odor** exposure, where mice were place in a large Plexiglas novel cage for 5 minutes, and exposed to bursts of air from a 50 ml syringe that was filled with a cotton swab tip dipped in fox urine (Buck Expert Inc., St. Benjamin, Que);

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7) **predator (cat) odor** exposure, where mice were place in a novel cage containing cat litter soiled with cat feces & urine, for 2 hours (this stressor was only administered once on each day);

8) **footshock,** where mice were individually placed in a footshock apparatus (4.5 x 5.0 x 5.0 cm) that was housed in a ventilated, sound attenuating chamber (30 x 55 x 50 cm). Each mouse was exposed to 15 shocks of 500 ms duration at 30 sec intervals (0.3 mA, 60 Hz, ac). The shock was delivered from the floor of each footshock chamber, consisting of 0.32 cm stainless steel rods spaced 1.0 cm apart, connected in series by neon bulbs;

9) **intraperitoneal injection** of 0.35 ml of sterile saline solution, with injections given on opposite sides of the abdomen on morning and afternoon stressor sessions;

10) **food deprivation,** where food was removed from mice for a single 15 hr period.

The final stressor was the presentation of 30 acoustic startle stimuli ranging from 75-115 dB in 10 dB increments, at 30 second intervals. Mice had not been exposed to this stressor prior to the final stressor day. The acoustic startle system (MED Associates Inc., St. Albans, Vermont) was housed in a ventilated, sound attenuating chamber (30 x 55 x 50 cm). In each chamber, a wideband speaker (1 – 16 kHz) provided the audio source for startle stimuli, as well as background noise. The presentation of stimuli was controlled by startle reflex software (version 4.21) (MED Associates Inc., St. Albans, Vermont). Mice were individually placed in a clear, cylindrical acrylic holder (4.45 x 5.7 cm), which was attached to a rectangular base that was fastened to the startle platform. Animals were allowed to acclimate to the startle chamber for a five-minute period. Low levels of ambient light were generated in each chamber by a 3 watt red filtered light, and

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background white noise with an intensity of 45 dB was maintained to minimize the impact of noise coming from outside of the chamber. Following the habituation period, each animal was presented five startle stimuli (50 ms pulse of white noise at 115 dB) at 30 second intervals, after which the 30 startle stimuli were randomly presented. At the end of the startle presentation, animals were immediately removed from the startle chamber and returned to their home cages.

Behavioural Assessments

During the latter part of the chronic stressor regimen, two additional stressors were applied during which behavioural measures were taken. These were applied only on a single occasion. Four additional animals that were not previously exposed to a stressor were assessed, serving as a comparison for chronically stressed animals. The following stressors were included as behavioural tests:

Forced swim (as previously described): The total time spent actively swimming, and the total time spent floating was recorded for 3 separate trials of 1 minute each, with a 1 minute waiting period in between trials.

Open-field emergence (6 minutes): mice were placed in a small darkened chamber that contained an entryway (7.5 x 7.5 cm) to a large square opaque field (80 x 80 x 60 cm). The latency to display the first stretch-attend response (i.e. wherein mice place their forelegs into the open-field, stretch forward and withdraw), the total number of stretch-attends, the latency to enter the open-field, and the total number of entries and exits from the small chamber was recorded.

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Tissue preparation and fixation

3 hours following the final stressor, mice were perfused intracardially with phosphate-buffered saline (PBS) and Lana's fixative (20% paraformaldehyde with saturated picric acid, in PBS). Brains were left in the Lana's solution for 24 hours, and were placed in a 30% sucrose cryoprotectant solution until they were ready to be processed. Brains were sectioned at 12 μm onto Fisherbrand **Superfrost Plus* glass slides, and were kept at -20°C until they were ready to be processed.

Immunohistochemistry

A barrier was created around the tissue sections using a liquid barrier pen that would dry onto the slides. During all washes, the slides were placed in a clear plastic chamber that contained a moist filter paper to maintain humidity during incubations. The liquid was pipetted onto each individual slide within the barrier. This procedure prevented the sections from coming off of the slides during washes. Sections were washed with PBS, 3 times for 5 minutes, followed by PBS with 0.2% Triton-X, 3 times for 5 minutes. The sections were then labeled with primary antibodies, incubating for approximately 20 hours at 4°C. Median eminence sections were double-labeled with anti-Guinea pig Arg8-vasopressin, at a 1:25000 dilution and anti-Rabbit CRH, at a 1:2000 dilution (Peninsula Laboratories, Inc.). As the CeA was present in all sections that contained the ME, there was no need for separate staining of CRH for this region. Thus, the dilution of the anti-Rabbit CRH primary antibody for the CeA was 1:2000. Prefrontal cortex sections were labeled with anti-Goat CRH receptor (RI & RII) at a dilution of 1:2000 (Santa Cruz Biotechnology, Inc.). All primary antibodies were diluted in

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antibody diluting buffer (DAKO Diagnostics Canada, Mississauga, ON). Another wash of PBS with 0.2% Triton-X was done 3 times for 10 minutes, followed by incubation with secondary antibodies. Median eminence and CeA sections were labeled with Alexa 488 Fluor labeled Goat anti-Guinea pig IgG, at a 1:500 dilution (Molecular Probes, Inc.) in PBS and Cy3-conjugated AffiniPure Donkey anti-Rabbit IgG, at a 1:3500 dilution (Jackson Immuno Research Laboratories, Inc.) in PBS. Prefrontal cortex sections were labeled with Cy3-conjugated AffiniPure Donkey anti-Goat IgG, at a 1:5000 dilution (Jackson Immuno Research Laboratories, Inc.) in PBS. Incubation for secondary antibodies took place at room temperature for 2 hours, followed by a wash with phosphate buffer with 0.2% Triton-X, 3 times for 10 minutes, and a final wash with an equilibration buffer for 5 minutes. All washes and incubations took place on an agitator to ensure thorough washes and adequate antibody binding. Slides were mounted with glass cover slips, in *SlowFade* Antifade with DAPI mounting medium (Molecular Probes, Inc.), and were sealed using nail polish. Slides were stored at -20°C and protected from light until they were ready to be imaged.

Image Acquisition and analysis

All images were taken with a Leica DMXRA wide-field fluorescence microscope with a 20x objective (Numerical aperture (N.A.) 0.5) having an optical resolution of 540 nm @ λ = 540 nm and 590 nm @ λ = 590 nm, and a 10x objective (N.A 0.3) having an optical resolution of 900 nm @ λ = 540 nm and 983 nm @ λ = 590 nm. The microscope was equipped with a Photometrics *CoolSnap fx* CCD camera (1300 x 1030 pixels), using Openlab software (Improvision). An additional magnification of 0.63 times at the camera

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resulted in each pixel being 0.53 μm at the 20x objective, and 1.06 μm at the 10x objective. A stack of 10 images, with a spacing of 0.2 μm in the z-axis (z-step) was taken. This was performed for each wavelength channel: 590 nm (Cy3), 540 nm (Alexa 488), and 450 nm (DAPI), for a total of 30 images for each ME picture, and 20 images for each CeA and PFC picture.

The stacks of wide-field images were then deconvolved using Exhaustive Photon Re-assignment (EPR) software (Scanalytics) to generate confocal stacks for quantitative analyses. The deconvolution procedure eliminated the distortion of light, created by the wide-field microscope. This was achieved by collecting light from a point source, to create an error function, called a point source function (PSF). The PSF was then used to calculate how the image was distorted, and reassign the distorted light to its theoretical point of origin, resulting in high-resolution confocal images. Fluorescently labeled Styrofoam beads (Molecular Probes) having a radius of 0.175 μm were used for the PSFs, as their size is smaller than the resolution of the objective, and therefore behave as point sources of light. Stacks of images of these beads were taken at each objective and each wavelength, ranging from the top of the focal plane to the bottom of the focal plane of the bead. The PSFs were then used to deconvolve the wide-field images obtained under the same z-step and objective. The resulting high-resolution confocal images were then used to determine the amount of co-localization of the two immuno-labeled peptides.

Image analysis was performed using IPlab software (Scanalytics). The stacks of ME images from each color channel were superimposed to create one frame from which the degree of co-localization of CRH (Cy3 labeled staining) and AVP (Alexa 488 labeled

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staining) could be determined. In a given region of interest (ROI), only the pixels within brightest 5% were used to co-localize the signals. Thus, in the case of the ME, CRH and AVP were considered to be co-localized in the pixels where the intensity of both of the signals were at least in the top 5% in terms of their intensities. These pixels were turned to a different color to visualize the peptide expression, a procedure called segmentation. The program was then able to quantify the number of pixels that were co-localized (or segmented) within the ROI. In the case of the oPFC and CeA, the signals were segmented at a user-determined level above background. Thus, only the signals that were at least 50% above background were quantified. The segmentation values for all three regions were expressed as a percentage of the total area of the ROI, and the percentage values obtained for each animal of each experimental group were statistically analyzed.

Statistical Analyses

Data were analyzed through a 2 (strain) x 3 (stressor condition) analysis of variance (ANOVA). Where appropriate (e.g., forced swim and startle), a mixed measures ANOVA was used. Post-hoc comparisons were conducted using the Newman-Keuls procedure.

Results

CRH receptor immunoreactivity in the prefrontal cortex

CRHr immunoreactivity was measured within four areas of the oPFC, corresponding to the agranular insular cortex (AI), lateral orbital cortex (LO), ventral orbital cortex (VO) and medial orbital cortex (MO), as seen in Figure 1. Similar patterns

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of CRHr immunoreactivity were detected within the four regions, thus only images for the MO are shown (Figure 2).

Within the AI region of the PFC, analysis of CRHr levels revealed that the Strain x Stressor Treatment interaction failed to reach statistical significance $F(1, 28) = 2.595$, $p = 0.0925$. Nevertheless, it is clear from Figure 3A that the two mouse strains responded to the acute stressor in an opposing manner. Specifically, basal levels of CRHr were lower in the C57BL/6ByJ strain relative to BALB/cByJ, but this was just shy of statistical significance. With acute and chronic stressor exposure BALB/cByJ mice showed a decrease of CRH receptor-ir (immunoreactivity). C57BL/6ByJ mice, in contrast showed an increase of CRH receptor-ir following acute stressor exposure, whereas the chronicity of the stressor had no effect.

Within the LO region of the PFC (Figure 3B), there was a significant Stressor treatment effect, $F(2, 29) = 6.495$, $p < 0.005$. Follow-up comparisons indicated that control BALB/cByJ and C57BL/6ByJ animals did not differ with respect to CRHr levels. The acute stressor provoked only a marginal non-significant decrease of CRHr levels within BALB/cByJ mice, and the opposite effect in C57BL/6ByJ mice relative to controls. In contrast, chronically stressed animals of both strains showed significantly lower levels of CRH receptor-ir did non-stressed mice, indicating the chronic stressor regimen indeed had a significant impact on CRHr levels.

As shown in Figure 3C, CRHr levels within the VO region of the PFC varied as a function of the Strain x Stressor treatment interaction, $F(2, 29) = 6.003$, $p < 0.05$. The follow-up comparisons indicated that two strains did not differ with respect to baseline levels of CRH receptor-ir. However, following the stressor treatments these strains were

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found to differ appreciably from one another. Specifically, among BALB/cByJ mice, both the acutely and chronically stressed animals showed a decrease of CRH receptor-ir relative to non-stressed controls, however the effect was only statistically significant among acutely stressed mice. Consistent with the pattern of CRHr levels seen in the LO region of the PFC, C57BL/6ByJ mice showed a moderate increase of CRH receptor-ir in response to the acute stressor treatment, and a slight decrease from the chronic stressor treatment. Moreover, chronically stressed mice showed significantly lower levels of CRH receptors than acutely stressed mice.

Within the MO region of the PFC, as seen in figure 3D and Figure 2, there was a significant Strain x Stressor treatment interaction with respect to CRH receptor-ir: $F(2, 29) = 7.211$, $p < 0.005$. Once again, mice of the two strains did differ with respect to basal CRH receptor-ir. However, the follow-up comparisons indicated that acute and chronic stressor exposure within BALB/cByJ mice provoked a significant decrease of CRH receptor-ir. In the C57BL/6ByJ strain however, the acute stressor treatment caused a significant increase of CRHr levels relative to control mice, but no change following chronic stressor exposure.

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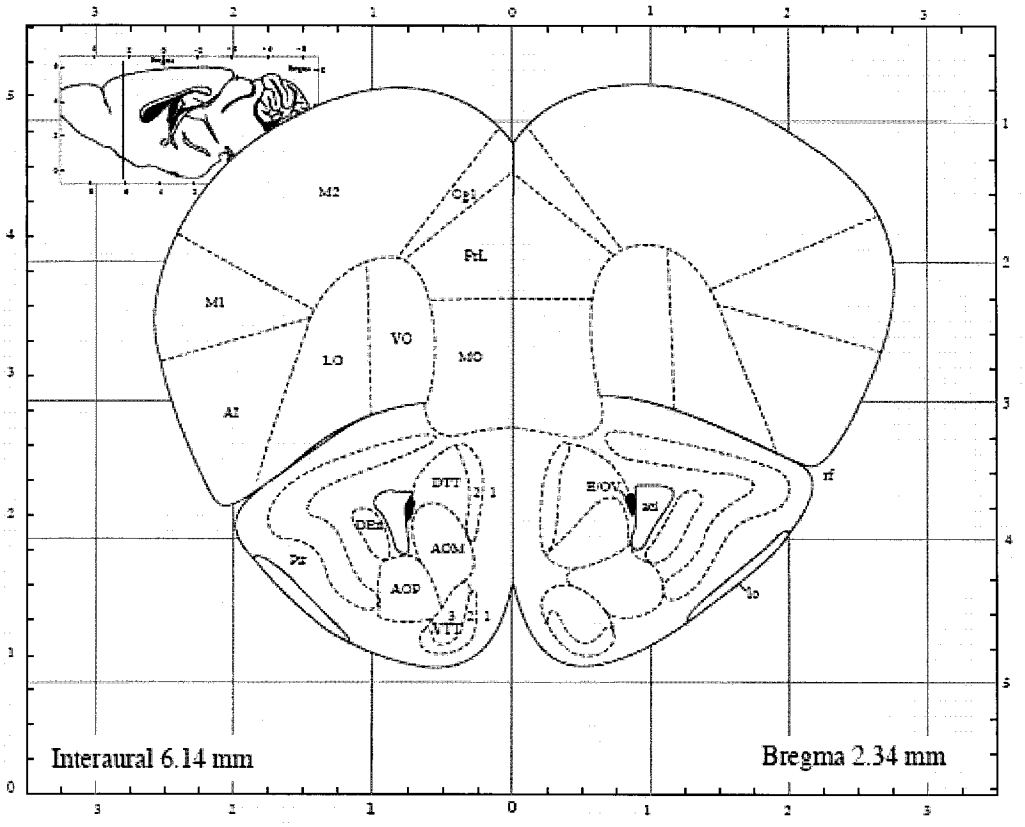

Figure 1. Coronal diagram from a mouse brain atlas showing the agranular insular (AI), lateral orbital (LO), ventral orbital (VO) and medial orbital (MO) areas of the prefrontal cortex at Bregma 2.34 mm (Paxinos & Franklin, 2001). The prefrontal cortical sections used in this experiment were taken at approximately the same Bregma value of this diagram.

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Figure 2. Deconvolved wide-field images of the medial orbital prefrontal cortex (20X magnification), stained with anti-Goat CRH receptor I & II antibody (red) and DAPI (blue). BALB/cByJ control (top left), C57BL/6ByJ control (top right), BALB/cByJ acute (middle left), C57BL/6ByJ acute (middle right), BALB/cByJ chronic (bottom left), C57BL/6ByJ chronic (bottom right). CRH receptor immunoreactivity is concentrated around the cell bodies (blue).

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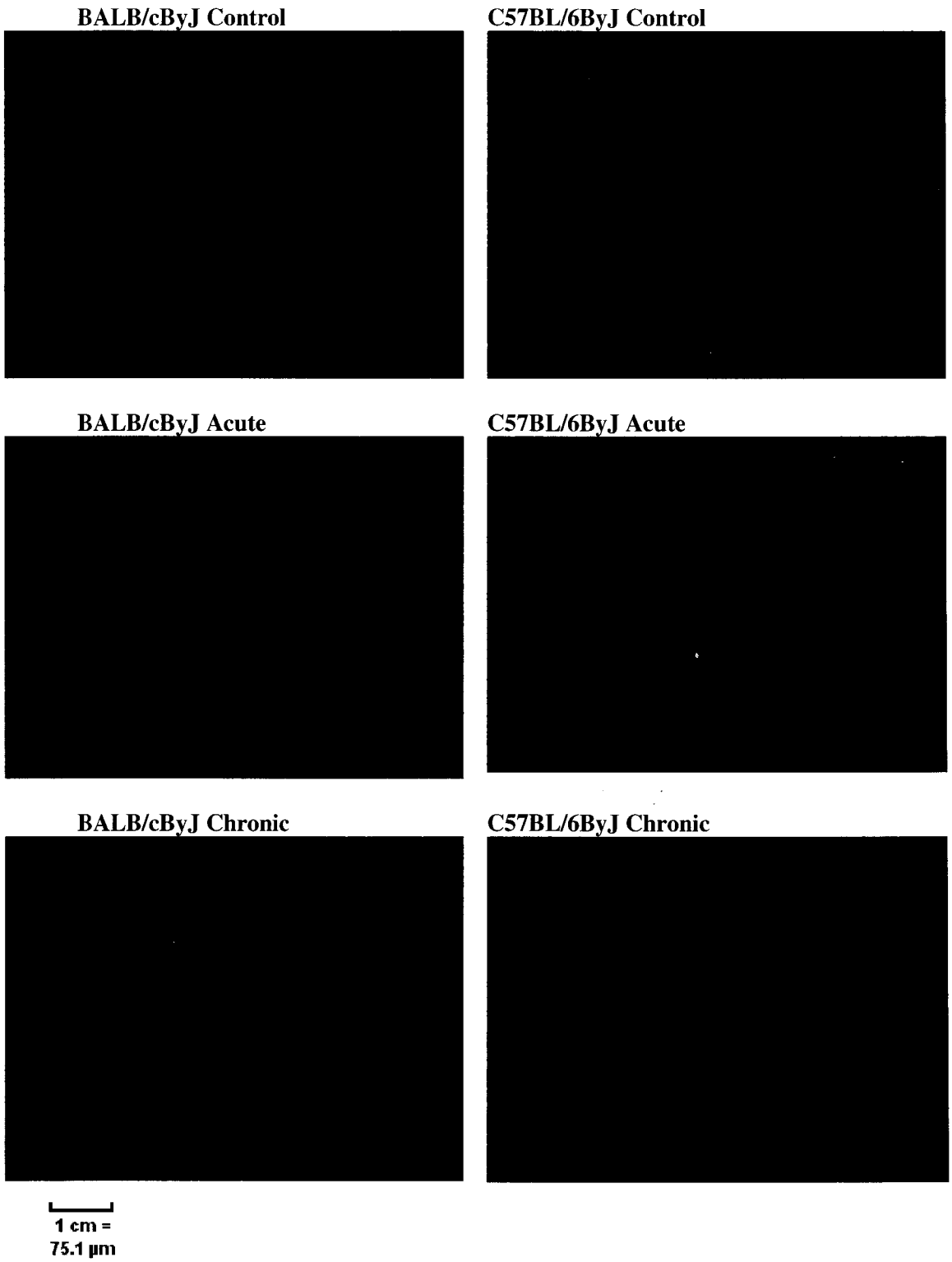

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Figure 3. Mean (± SEM) levels of CRH receptor immunoreactivity within the four regions of the PFC (Graphs A, B, C, D) in BALB/cByJ and C57BL/6ByJ mice, as a function of the stressor treatment. Levels of CRH receptor immunoreactivity are expressed as a percentage of the expression within a region of interest (ROI). * $p < .05$; ** $p < 0.01$; *** $p < 0.001$ relative to non-stressed mice of the same strain.

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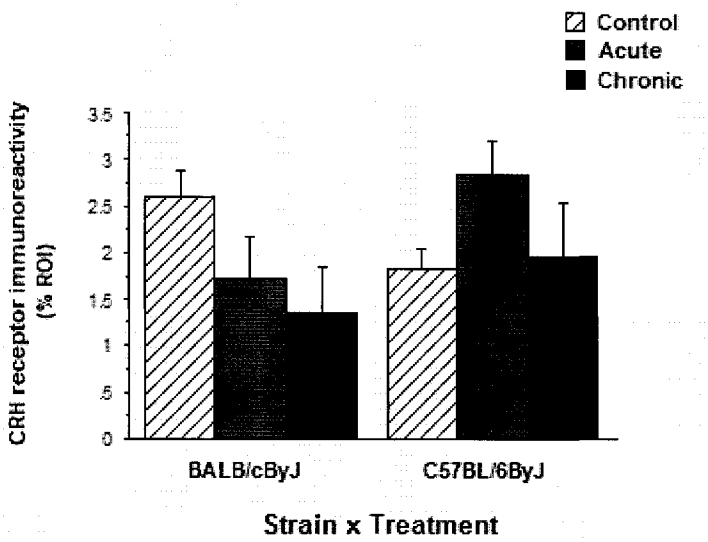
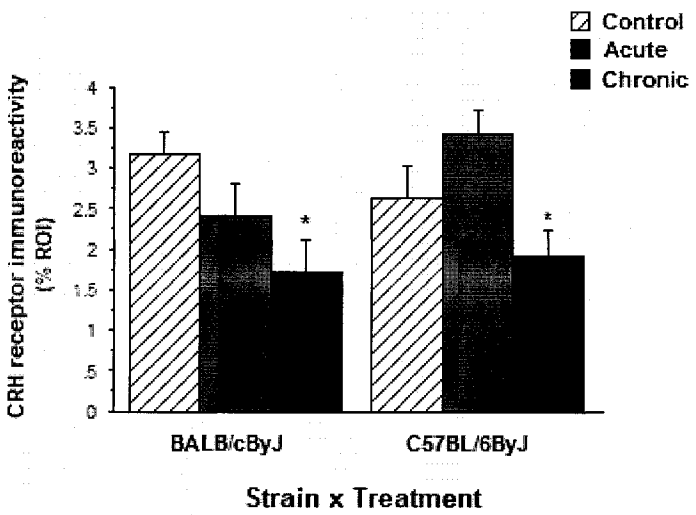
A Agranular Insular PFC



B Lateral Orbital PFC



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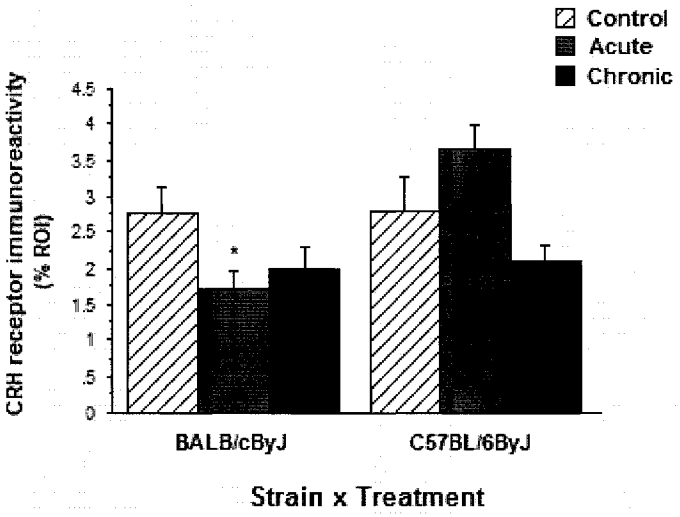
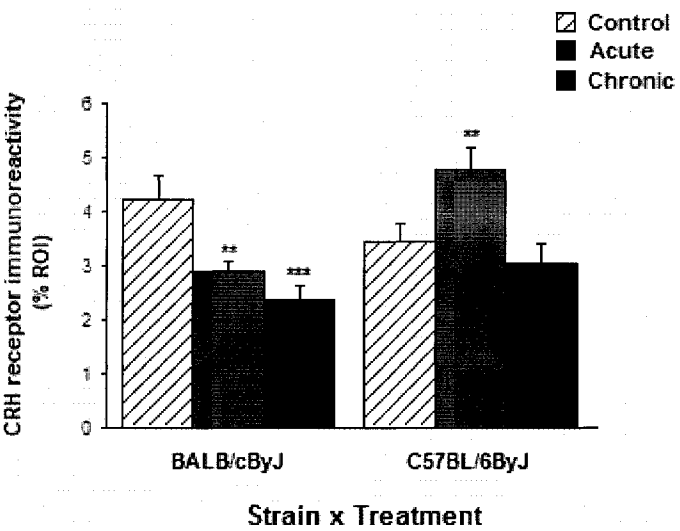
C Ventral Orbital PFC



D Medial Orbital PFC



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Co-localization of CRH and AVP in the external zone of the median eminence

The acute and chronic stressor treatments did not have a significant impact on the co-localization of CRH and AVP within the ME (Figures 4 and 6). There was a small, but significant elevation of CRH/AVP co-localization in C57BL/6ByJ relative to the BALB/cByJ mice, $F(2, 51) = 4.139$, $p < 0.05$. This effect however, was negligible, given that it was not present in control animals, and was only marginally present in acute and chronic groups, with C57BL/6ByJ mice having slightly higher levels than BALB/cByJ mice. Moreover, when total CRH was measured separately, the two strains did not differ with respect to basal levels. Within BALB/cByJ mice, the acute and chronic stressor treatments had no effect on levels of CRH-ir, however, the chronic stressor treatment elicited a marginal non-significant increase of CRH levels in C57BL/6ByJ (Figure 6B).

CRH peptide immunoreactivity within the central nucleus of the amygdala

Within the CeA, the stressor treatment significantly affected CRH levels in a strain-specific manner, as indicated by the significant Strain x Stressor treatment interaction, $F(2, 26) = 4.933$, $p = 0.015$ (Figures 8 and 9). The follow-up comparisons indicated that among control animals, BALB/cByJ mice showed significantly higher CRH-ir than did C57BL/6ByJ mice. It was also found that within the acute and chronically stressed BALB/cByJ mice a non-significant increase of CRH peptide-ir occurred; however the acute and chronically stressed mice did not differ from one another. In contrast, within mice of the C57BL/6ByJ strain, the acute and chronic stressor exposure elicited a non-significant decline of CRH levels, and again, these groups did not differ from each other. As a result of these changes, the difference between the strains

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observed in the absence of a stressor, were entirely eliminated by both the acute and chronic stressor treatments.

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Figure 4. Deconvolved wide-field images of the median eminence (20X magnification) stained with anti-rabbit CRH antibody (red), anti-guinea pig AVP antibody (green), and DAPI (blue). Yellow pixels represent the co-localization of the red and green signals. Only the pixels within brightest 5% were used to co-localize the signals. BALB/cByJ control (top left), C57BL/6ByJ control (top right), BALB/cByJ acute (middle left), C57BL/6ByJ acute (middle right), BALB/cByJ chronic (bottom left), C57BL/6ByJ chronic (bottom right).

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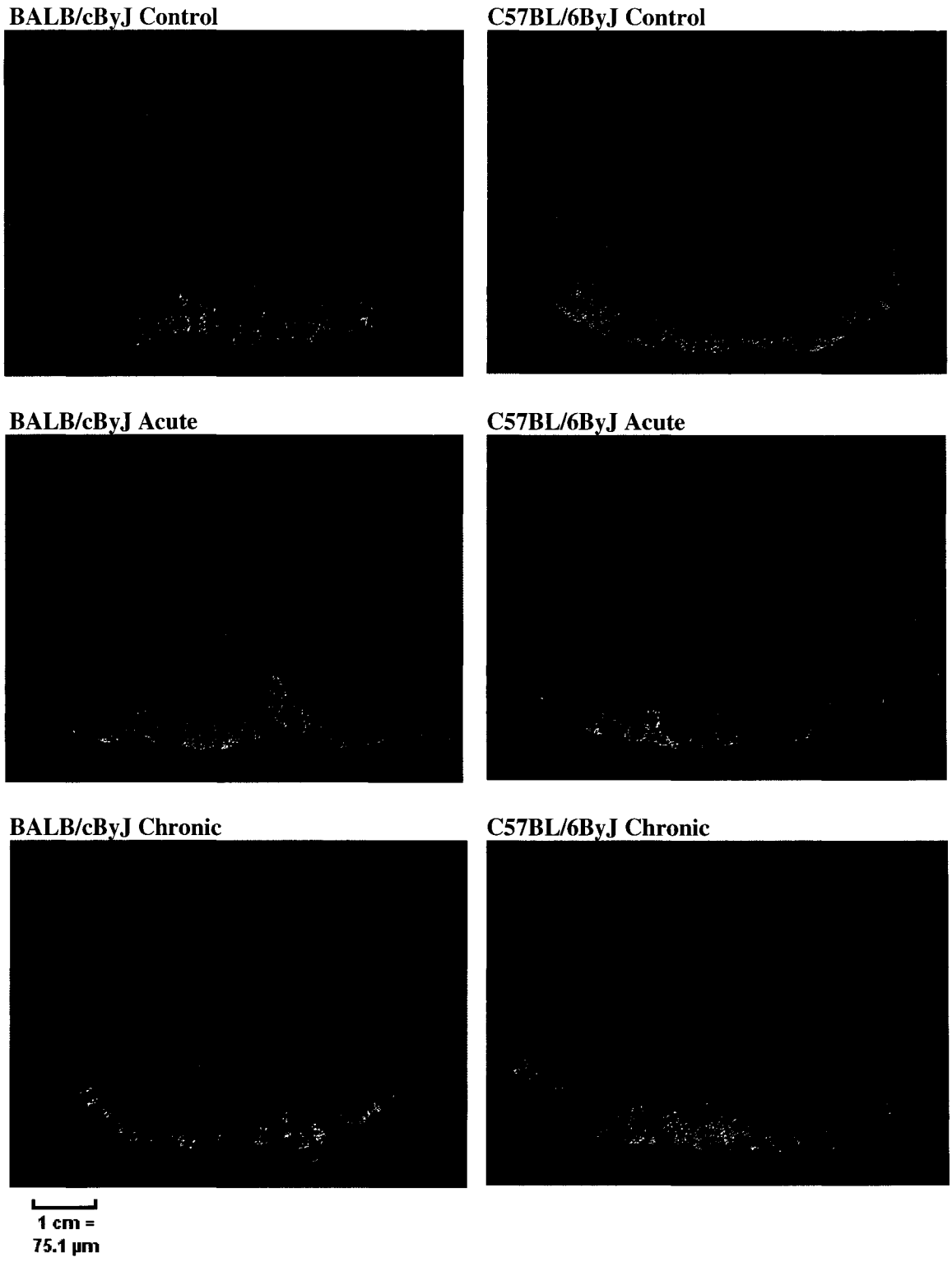

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Figure 5. (A) Deconvolved image of the ME showing red channel only (CRH staining). (B) Deconvolved image of the ME showing green channel only (AVP staining). (C) Combined image of A and B. Pictures of different color channels are acquired separately, then combined for analysis of co-localized signals. (D) Non-deconvolved image of the ME. Notice how the signals are blurred, compared to the deconvolved images.

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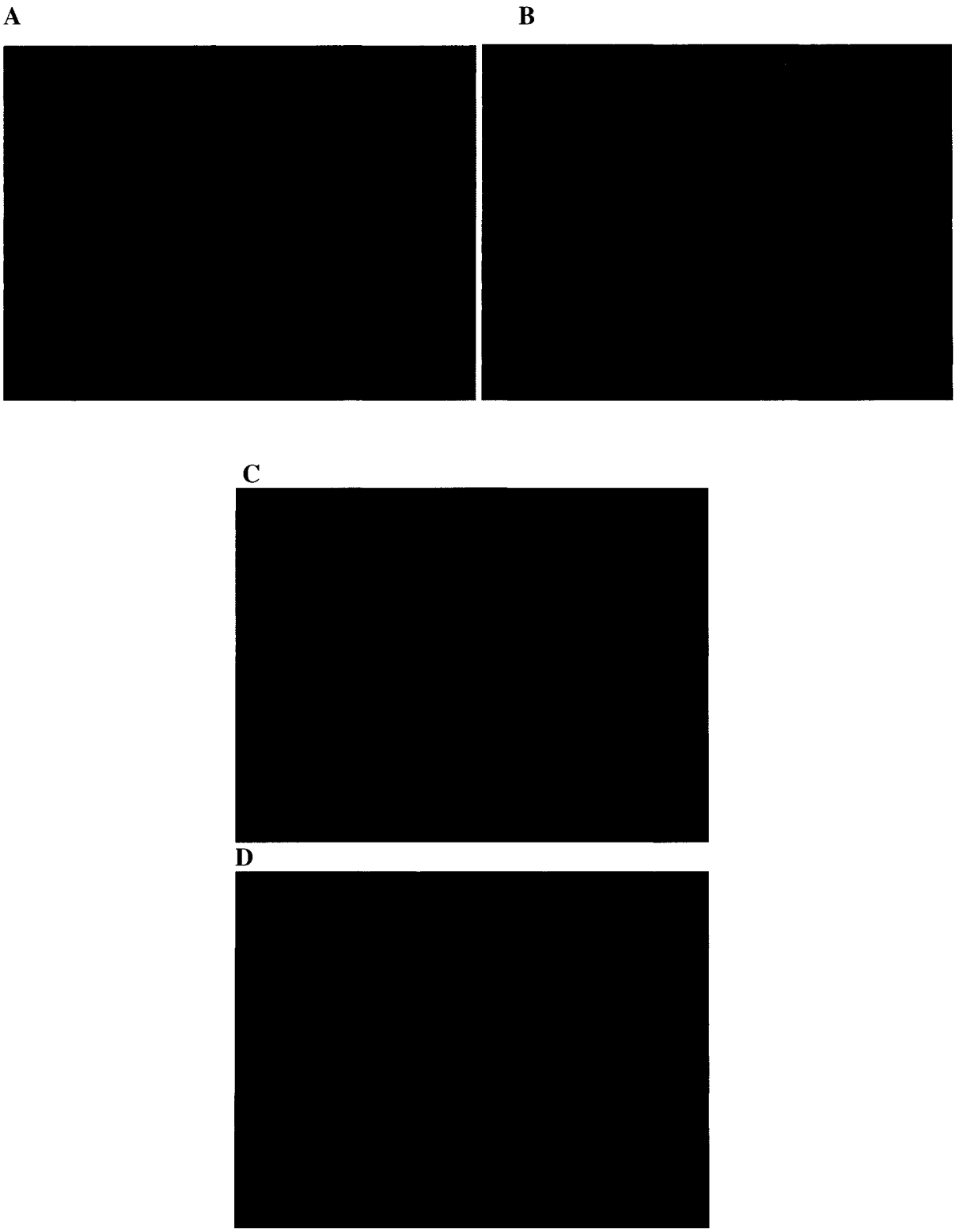

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Median Eminence

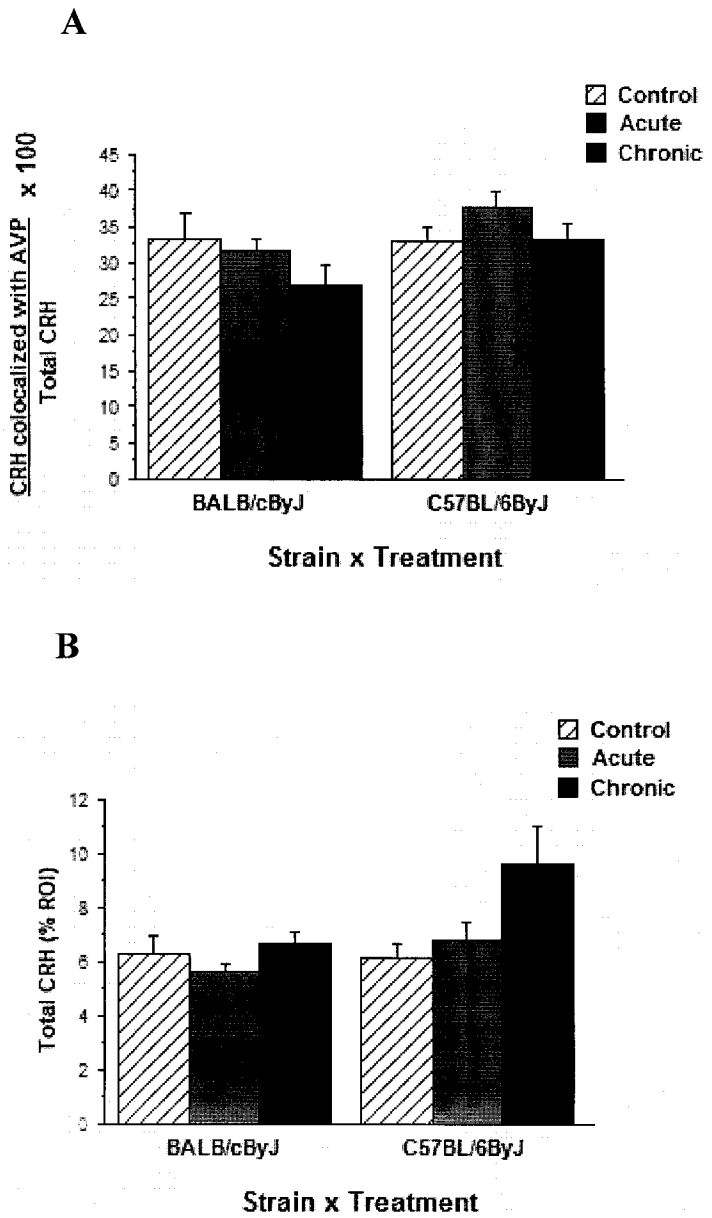

Figure 6. Mean (± SEM) levels of CRH/AVP peptide co-localization within the external zone of the median eminence in BALB/cByJ and C57BL/6ByJ mice as a function of stressor treatment. Values are expressed as a percentage of CRH that is co-localized with AVP, relative to total CRH levels within the area.

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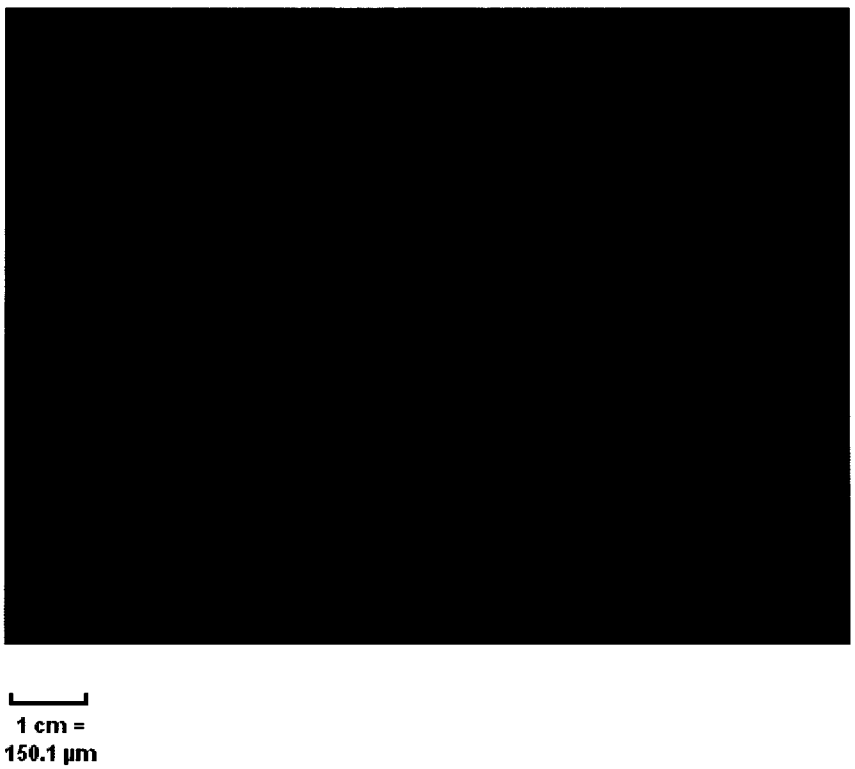

Figure 7. Wide-field image of the central nucleus of the amygdala, stained with anti-rabbit CRH antibody, taken at a 10X magnification.

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Figure 8. Deconvolved wide-field images of the central nucleus of the amygdala (20X magnification), stained with anti-rabbit CRH peptide antibody (red) and DAPI (blue). BALB/cByJ control (top left), C57BL/6ByJ control (top right), BALB/cByJ acute (middle left), C57BL/6ByJ acute (middle right), BALB/cByJ chronic (bottom left), C57BL/6ByJ chronic (bottom right).

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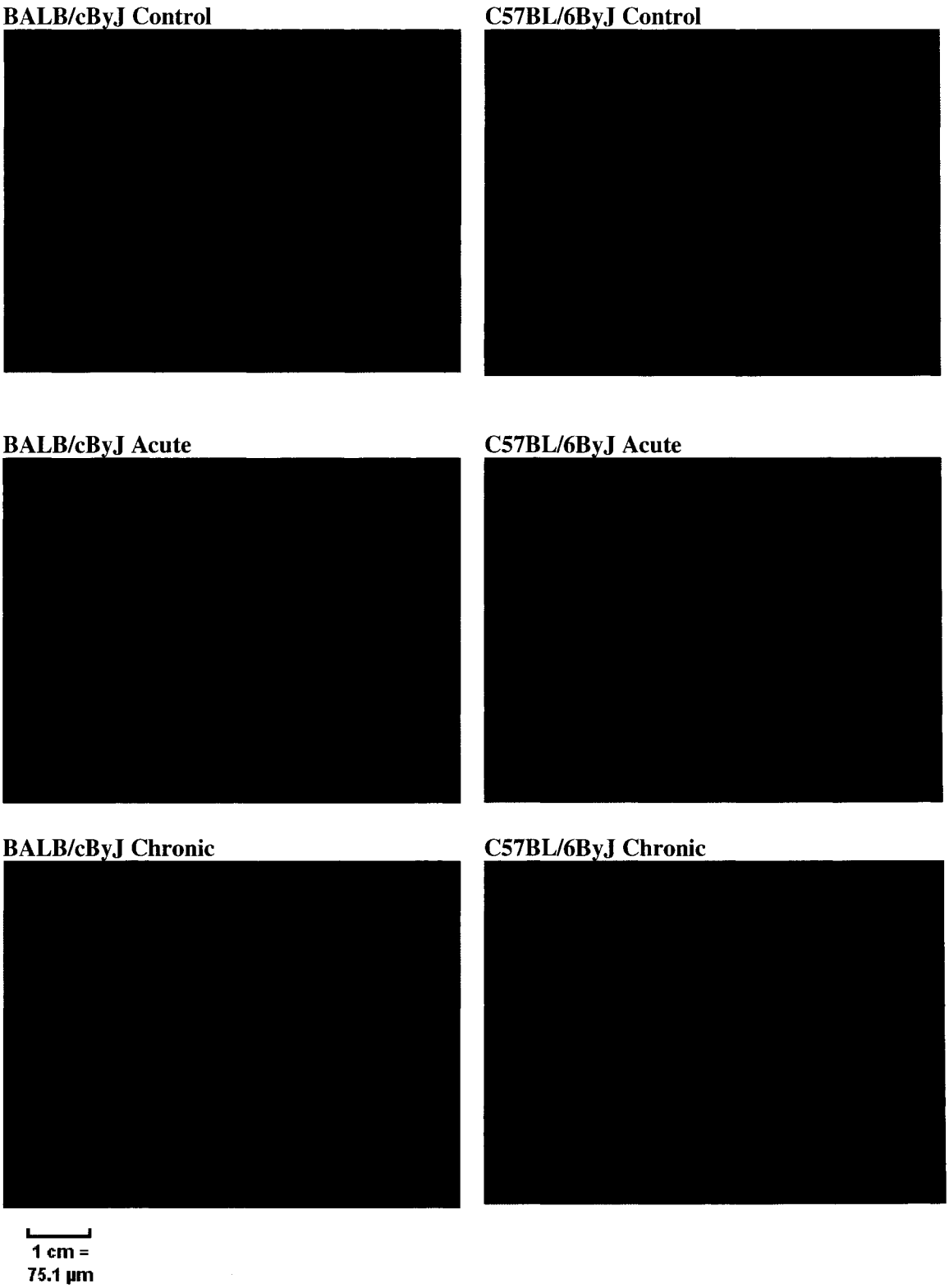

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Central Nucleus of the Amygdala

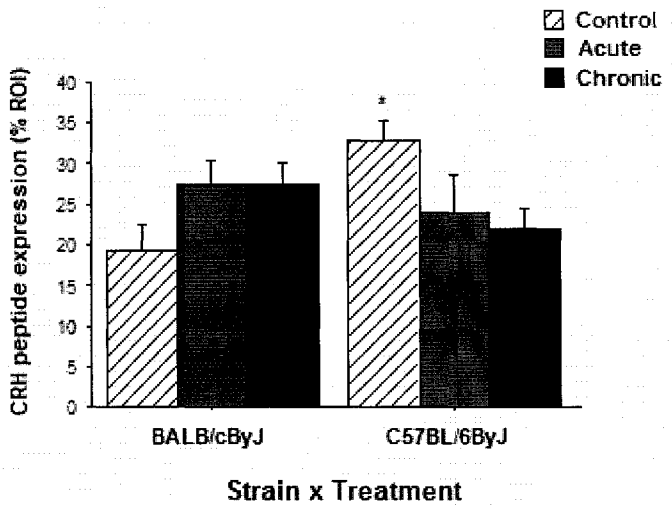

Figure 9. Mean (± SEM) levels of CRH peptide expression within the central nucleus of the amygdala in BALB/cByJ and C57BL/6ByJ mice, as a function of stressor treatment. Values are expressed as a percentage of the expression within a region of interest.

* $p < .05$

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Behavioural effects of acute and chronic stressor exposure

Analyses of the behavioural tests, revealed that the stressors differentially affected the two mouse strains. In a forced swim test, as depicted in Figure 10, the amount of time that mice spent actively swimming varied as a function of the Strain x Stressor treatment x Trials interaction $F(2, 54) = 11.134$, $p < 0.0001$. The follow-up tests indicated that on the initial trial the BALB/cByJ and C57BL/6ByJ control animals did not differ significantly from each other with respect to the levels of active swimming. While levels of active swimming in C57BL/6ByJ control mice declined significantly over the three trials, the high levels persisted within BALB/cByJ control mice. Chronically stressed C57BL/6ByJ mice initially displayed somewhat lower levels of active swimming relative the C57BL/6ByJ control animals, and interestingly, these levels increased significantly over the three trials. In contrast, BALB/cByJ mice that were chronically stressed displayed a precipitous decline of active swimming over the three swim trials, and this finding was statistically significant.

In the open-field emergence test (Figure 11), the latency to leave the dark box was significantly higher in BALB/cByJ than C57BL/6ByJ mice, $F(1, 27) = 36.859$, $p < 0.0001$. Similarly, the total number of stretch attend responses was significantly higher in the BALB/cByJ mice, $F(1, 27) = 19.445$, $p < 0.0001$. Conversely, the total time spent exploring within the open-field was significantly higher in C57BL/6ByJ than BALB/cByJ mice, $F(1, 27) = 107.427$, $p < 0.0001$. The acute or chronic stressor treatments did not, however, impact any of these behaviours.

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Finally, the startle amplitude was found to vary as a function of the noise intensity, $F(4, 84) = 34.11$, $p < .01$ (Figure 12). This was the case in both strains, wherein a marked increase of startle amplitude was apparent at the highest amplitude. The C57BL/6ByJ mice that had not previously been stressed exhibited a startle response 30-40% lower than that of the chronically stressed mice of the same strain or of similarly treated BALB/cByJ mice. However, the small N did not permit sufficient power to detect a significant interaction.

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Forced Swim Test

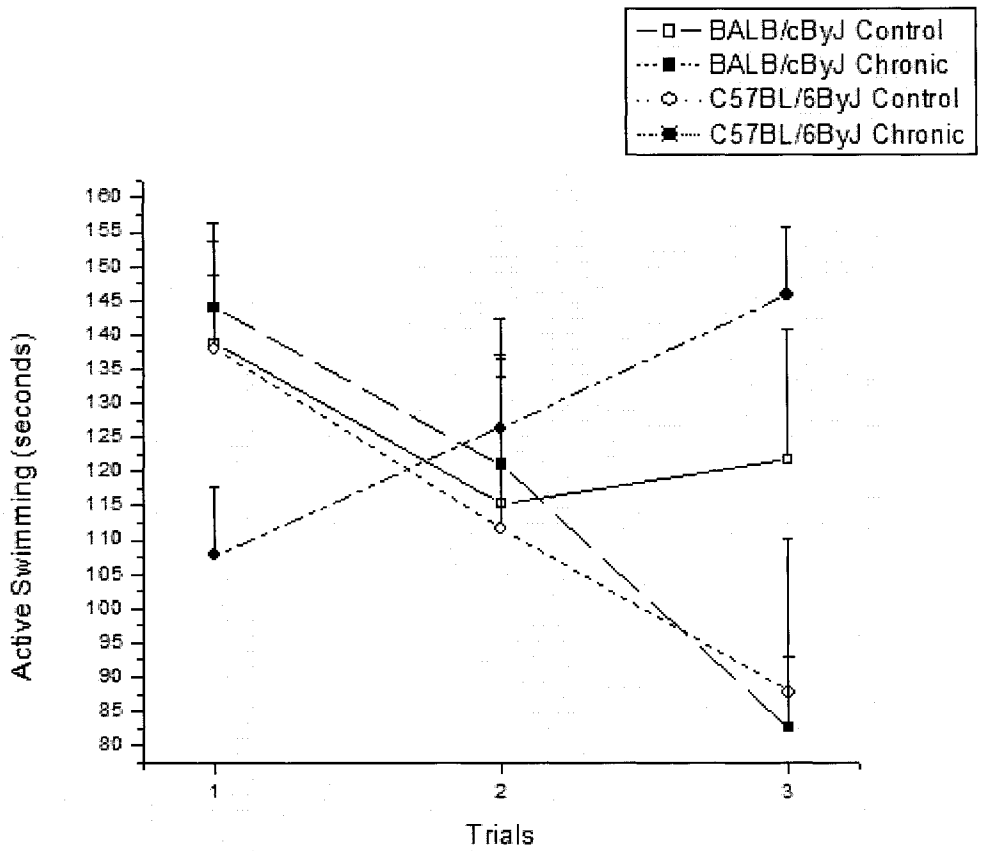

Figure 10. Mean (± SEM) time (seconds) engaged in active swimming on three successive 3-minute trials among BALB/cByJ and C57BL/6ByJ mice that had been exposed to a chronic stressor regimen, or that had no previous stressor exposure.

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Figure 11. Mean (± SEM) latency to emerge from the dark box into the open-field (top), total time spent exploring in the open-field (middle), and total number of stretch attends (bottom) displayed in an open-field emergence test among BALB/cByJ and C57BL/6ByJ mice that had been exposed to a chronic stressor regimen, or that had no previous stressor exposure.

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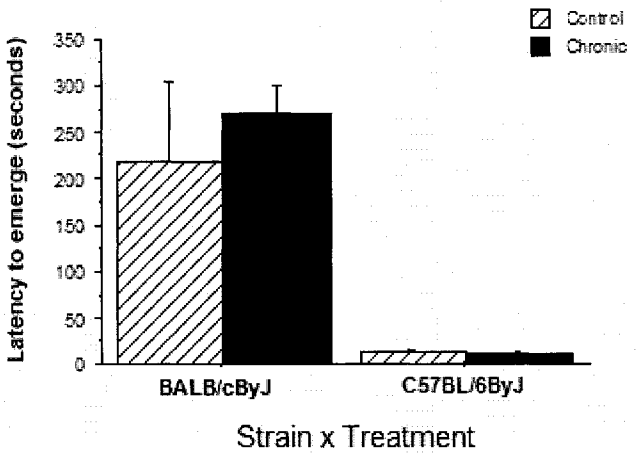
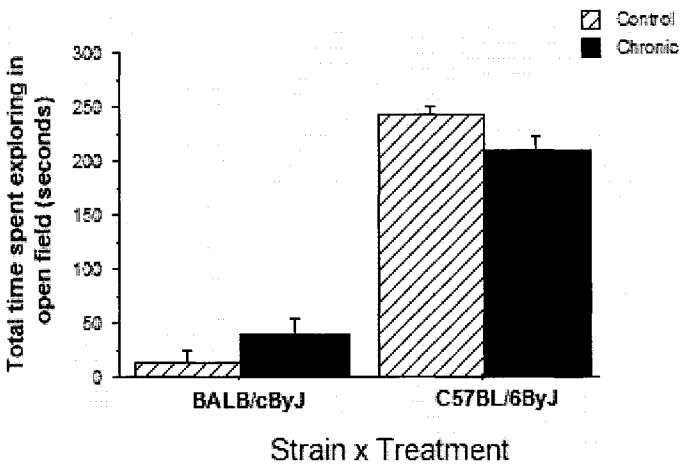
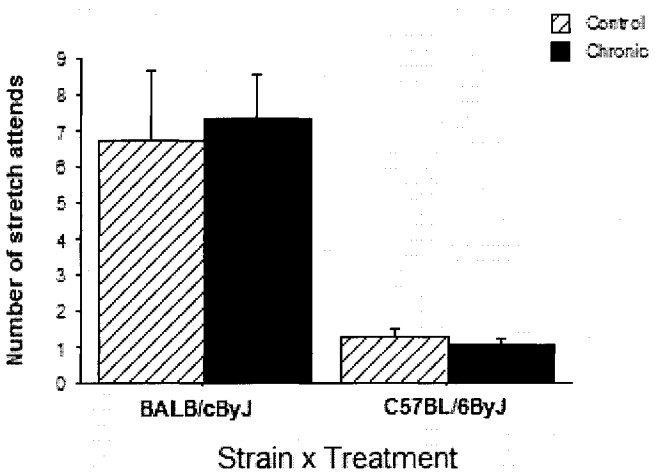
Open-field Emergence Test







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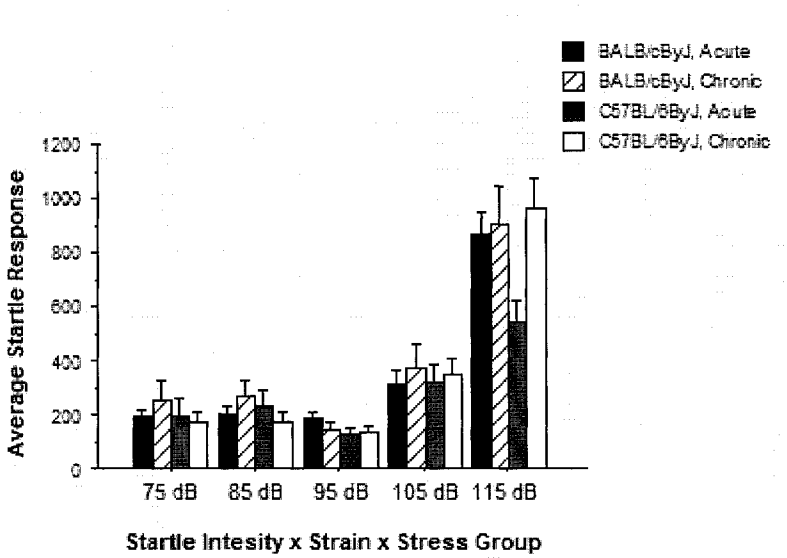

Figure 12. Mean (± SEM) startle responses averaged over trials (and startle intensities), in BALB/cByJ and C57BL/6ByJ mice that were either chronically stressed, or had no previous stressor exposure (belonging to the acute stress group). Mice had been exposed to 30 startle trials comprising 5 different intensities.

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Discussion

Results from the present investigation paralleled previous research in BALB/cByJ and C57BL/6ByJ mice with respect to their differential reactivity to stressors, and provide new evidence into the neuroendocrine basis for these differences. The following sections provide a discussion of the differences of CRH receptor expression within the oPFC, CRH peptide expression within the CeA, and CRH/AVP co-localization within the ME in response to acute and chronic stressor exposure.

Prefrontal cortical CRH receptor expression in BALB/cByJ and C57BL/6ByJ mice

It is well documented that certain areas of the PFC, particularly the medial division, provide inhibitory control over the HPA axis, suppressing HPA responses to various stressors (Brake et al., 2000; Crane et al., 2003; Diorio et al., 1993; Sulivan & Gratton, 1999). Results from the present investigation are consistent with this perspective, supporting the possibility that this outcome may be mediated, in part, by regulation of CRH receptors, but in this case within the orbital division of the PFC. However, as strains of mice differ with respect to CRH and CRH receptors, the relative contribution of this peptide to HPA functioning may be strain-dependent. Specifically, within all four areas of the oPFC that were measured, the stressor resilient C57BL/6ByJ mice showed an increase of CRH receptor-ir (immunoreactivity) in response to an acute stressor.

It was hypothesized that a compensatory up-regulation of CRH receptors within the oPFC would increase the inhibitory effects on HPA activity, notably, attenuating CRH release within the PVN, hence resulting in a more blunted GC response to the

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stressor. In contrast to C57BL/6ByJ mice, it was observed that BALB/cByJ mice responded to the acute stressor by a significant decrease of CRH receptor-ir in the same regions within the PFC. Down-regulation of CRH receptors in the oPFC would presumably decrease the inhibition over HPA activity, resulting in exacerbated or sustained GC responses to the stressor. Indeed, differences of GC responses to a variety of stressors have consistently been reported in these strains. BALB/cByJ mice exhibit greater increases of plasma CORT and ACTH to a variety of psychogenic and neurogenic insults (excluding predator-related cues) relative to the C57BL/6ByJ strain, and this corresponded with the seemingly greater anxiety in the BALB/cByJ mice (Anisman et al., 2001; Anisman et al., 1998). The diametrically opposite stressor-elicited CRH outcomes seen in BALB/cByJ and C57BL/6ByJ mice are not restricted to the PFC, but have also been reported within the PVN (Anisman et al., 1998).

Levels of CRH receptor-ir within chronically stressed C57BL/6ByJ mice were lower than among acutely stressed mice in each of the four oPFC regions, and were comparable to or modestly lower than among non-stressed mice. It appears that while acute stressors provoke an up-regulation of orbital prefrontal cortical CRH receptors within this strain, when the stressor was applied on a more protracted basis, a compensatory down-regulation of the response to the stressor was observed, such that CRH receptor levels returned to control levels. This can be interpreted as a compensatory mechanism that ensures an adequate HPA response to further anticipated stressors. Alternatively, it is possible that with sufficient stressor exposure, CRH receptor-ir would decline to levels of BALB/cByJ mice, reflecting allostatic overload, even among hardy C57BL/6ByJ mice. From this perspective, the levels of CRH receptor-ir observed after 7

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weeks of stressor exposure represents a point on a continuum of declining receptors that would be still more pronounced with continued stressor exposure. In effect, while the resiliency of this strain may offer protection to a certain degree, with sustained exposure, stressors are likely to exert a negative impact even against a genetic background that affords resiliency.

As previously mentioned, acutely stressed BALB/cByJ mice showed CRH receptor-ir levels that were significantly lower than those of control mice, and this effect was maintained following the chronic stressor treatment. Only a modest decline (or none at all) was observed when comparing chronically stressed to acutely stressed mice. At first blush, these data might be taken to suggest that the chronic stressor regimen had little effect on CRH receptor regulation within the oPFC in this strain. This was somewhat surprising, as previous studies that assessed the effects of chronic stressors revealed markedly greater behavioural signs of depression and anxiety following a chronic stressor regimen in BALB/cByJ than in C57BL/6ByJ mice (Tannenbaum & Anisman, 2002). As the PFC modulates some of the behavioural and cognitive disturbances associated with stressors (Mizoguchi et al., 2004), it was expected that CRH receptor changes in BALB/cByJ mice would likewise become more pronounced following the chronic stressor regimen.

Owing to the persistent CRH receptor down-regulation within the oPFC of BALB/cByJ mice (as a result of the limited HPA inhibition), it would be expected that circulating GC levels would be sustained over the chronic stressor period in this strain, potentially leading to behavioural symptoms of depression and anxiety. In this regard, it has been argued that prolonged release of GCs has damaging effects, including

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hippocampal cell death, leading to affective disorders such as depression and anxiety (Chrousos, 1998; McEwen, 2000). Of course, it is equally possible that the depressive-like symptoms resulting from the chronic stressor regimen are not mediated by CRH receptors alone, and thus it might be expected that the behavioural and CRH changes would not parallel one another. Indeed, it is well known that stressors influence monoamine functioning, and, it was previously reported that following acute and chronic stressors BALB/cByJ mice exhibited more widespread and marked changes of monoamine utilization relative to C57BL/6ByJ mice (Tannenbaum & Anisman, 2002).

The bulk of the literature concerning changes of CRH receptors, and particularly CRH_1 receptors, following acute and chronic stressors, have involved analyses of nuclei within the hypothalamus, notably the PVN (Cook, 2004; Imaki et al., 2001; Imaki et al., 1996; Jiang et al., 2004; Makino et al., 1999; Rivest et al., 1995), and little information is available regarding stressor-elicited CRH receptor changes within the PFC. As the PFC has been shown to exert important regulatory effects on the HPA system, and has been implicated in executive functioning, it would be useful to determine how CRH and CRH receptors in this area behave in response to chronic stressors.

Several lines of evidence point to a role for the PFC, and specifically CRH within the PFC, in subserving stressor-induced cognitive and emotional behaviour. The finding that prefrontal cortical deficits are prominent in a number of mental disorders that are exacerbated by stressors, particularly depression and anxiety-related illnesses, supports this view (Mazure, 1995). In line with this position, it was reported that CRH_1 mRNA levels were reduced in the frontopolar cortex of suicide victims, despite elevated levels of CRH in this region (Merali et al., 2004a). These findings are supported by evidence of

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down-regulation of CRH binding sites in the frontal cortex of depressive suicides, relative to non-depressed controls (Nemeroff et al., 1988). Results from the present investigation similarly revealed that acute and chronic stressors, in fact, affected CRH receptors within the PFC, and thus might contribute to inhibitory effects on HPA activity.

Stressor-related effects within the orbital division of the PFC

Most studies assessing neurochemical changes within the PFC in response to stressors have been concerned with the medial division of this region. As will be recalled, lesion studies indicated that damage to the mPFC produced marked HPA responses to stressors, indicating its inhibitory role over HPA activity (Diorio et al., 1993). Far fewer studies have examined changes within the oPFC, even though this region too, has been linked to the effects of stressors and depression. Studies in humans reported abnormally elevated cerebral blood flow (CBF) and metabolism within the oPFC in patients with major depression (Drevets, 2000). Moreover, these measures were found to increase during experimentally induced sadness and anxiety, and to decrease following antidepressant treatment. Similarly, regional brain functioning within the oPFC was positively correlated with levels of anxiety (Grachev & Apkarian, 2000). Interestingly, PET images acquired in subjects that were repeatedly exposed to phobic stimuli revealed that initial exposure to the phobic stimuli (when fear was at a peak level) yielded no changes in CBF within the oPFC, but showed progressive increases in CBF during subsequent exposures, as subjects habituated to the fearful stimuli (Drevets et al., 1995). Although it is still premature to elucidate whether the oPFC plays a role similar to the mPFC with respect to HPA activity (i.e. inhibitory regulation), these findings are

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consistent with such a possibility. Moreover, the expression of CRH receptors within the oPFC, as found in our investigation, implies some relationship to the HPA axis. Connections with the amygdala and hypothalamus further implicate the oPFC in regulation of stress responses (Drevets, 2000), although it remains to be determined whether the oPFC and mPFC play parallel or differing roles with respect to stressor reactivity and HPA regulation. We are currently investigating CRH receptor changes in the mPFC within the same set of animals. Results from this will further contribute to our understanding of the roles of these two prefrontal cortical regions in the regulation of HPA activity.

CRH receptor subtypes and behavioural anxiety

The present investigation assessed CRH receptor-ir using an antibody that binds to both CRH_1 and CRH_2 receptors, and thus it was not possible to distinguish which receptor subtype was associated with the changes that were seen. However, numerous reports have concluded that the two receptor subtypes have pharmacologically and physiologically distinct functions, thus leading to behaviourally distinct actions. CRH_1 has generally been regarded as the primary mediator of the effects of CRH on the HPA system, and has particularly been associated with the stress response (Keck et al., 2005). It has consistently been reported that CRH_1 knockout mice display decreased anxiety, as well as an impaired neuroendocrine response to stressors, indicating that CRH_1 is critical in mediating behavioural as well as neuroendocrine responses to stressors (Bale et al., 2002; Liebsch et al., 1995; Skutella et al., 1998; Smith et al., 1998). Interestingly, however, in a conditional knockout mouse line, where CRH_1 was inactivated within

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forebrain and limbic structures, while leaving the HPA system intact, a significantly elevated CRH and CORT response was observed following exposure to a stressor (Muller et al., 2003). These data indicate a role for limbic (e.g. PFC, amygdala) CRH_1 in modulating anxiety that is independent of HPA functioning.

In contrast to CRH_1, CRH_2 receptors have generally been linked to the regulation of feeding behaviour (Bale et al., 2000; Keck et al., 2005; Spina et al., 1996; Wang et al., 2001). Increasing evidence, however, has pointed to a role for CRH_2 in mediating some anxiety-related behaviours. Mice deficient of CRH_2 (through infusion of CRH_2 antisense oligonucleotides) exhibit hypersensitivity to stressors, displaying increased signs of anxiety (Bale et al., 2000; Isogawa et al., 2003; Kishimoto et al., 2000). As well, CRH_2 knockout mice displayed similar anxiogenic-like behaviour when exposed to open-field emergence and elevated plus maze stressors, and heightened ACTH and CORT responses when exposed to restraint stress (Bale et al., 2000). These data indicate the possibility of opposing roles for CRH_1 and CRH_2 receptors, although conflicting data preclude a definitive conclusion in this regard.

It has been reported that antagonism of CRH_2 receptors can result in anxiolytic or anxiogenic-like effects, depending on the location of the receptor. For example, acute antagonism of CRH_2 within the lateral septum resulted in a reduction of shock-induced freezing (Bakshi et al., 2002). While these findings may appear paradoxical, it is suggested that the anxiolytic versus anxiogenic properties of CRH_2 operate at different time frames following stressor exposure (Keck et al., 2005). Furthermore, it was shown that female double mutant mice (lacking both CRH_1 and CRH_2 receptors) displayed diminished anxiety-like behaviour, yet the males showed significantly greater anxiety

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than females (Bale et al., 2002). Thus, the relative contribution of CRH_1 and CRH_2 receptors to anxiety appears to vary with gender, along with the characteristics and time course of the stressor. While it is still premature to elucidate whether the two receptor subtypes have parallel roles, or work in opposition, it seems that both receptors are important mediators of the stress response. It was suggested that CRH_1 might subserve the initial, rapid HPA activation in response to a stressor, while CRH_2 might mediate the subsequent behavioural adaptation (de Kloet, 2003). It will therefore be important to distinguish which of the two receptor subtypes were affected by stressor exposure in the present investigation.

It ought to be considered that the abundance of the two CRH receptor subtypes is, to a considerable extent, regionally specific. CRH_1 has been reported to be more abundant in the cortex of rats and mice, particularly in frontal cortical areas, whereas CRH_2 expression is generally confined to subcortical structures (Chalmers et al., 1995; Eghbal-Ahmadi et al., 1998; Pett et al., 2000). These findings raise the possibility that the changes of CRH receptor expression within the PFC that were seen in the present investigation likely reflected changes of the CRH_1 receptor subtype. While there are many stressor-sensitive regions that express both receptor subtypes, their distribution provisionally suggests a fundamental role for the CRH_1 receptors in mediating stressor-elicited effects within the PFC. Nonetheless, it would be necessary to repeat the present investigation using specific antibodies for CRH_1 and CRH_2 in order to discern which of the two receptor subtypes was affected by stressor exposure within the PFC.

Currently, data are unavailable concerning differences in the levels of the two CRH receptor subtypes in the C57BL/6ByJ and BALB/cByJ strains. Perhaps an

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abundance of CRH_1 and/or a deficiency of CRH_2 within the BALB/cByJ strain may contribute to the increase of behavioural anxiety and stressor reactivity that is typically seen in this strain. In general, the present investigation indicated that BALB/cByJ mice displayed higher basal levels of CRH receptor-ir within some areas of the PFC, although this finding was not statistically significant. It is uncertain, however, whether the strains differed with respect to the abundance of the CRH_1 and CRH_2 receptor subtypes.

Stressor-induced CRH expression in the CeA

While the stressor treatment provoked only non-significant changes of CRH expression within the CeA, they are still worth considering, particularly because of the strain differences that were observed. The acute and chronic stressors induced an increase of CRH-ir within BALB/cByJ mice, but provoked the opposite effect in C57BL/6ByJ mice. As a result, the strains differed significantly following the stressor treatments. It may be that these modest, but significant effects, accounted for the strain differences in anxiety often seen following stressor exposure. Increases of CRH content and CRH mRNA in the CeA following acute stressors have consistently been reported (Cook et al., 2004; Hsu et al., 1998; Makino et al., 1999; Merali et al., 1998). It appears that the nature of the stressor may have a considerable impact on such outcomes. Indeed, it has been suggested that the amygdala is involved in the evaluation and emotional appraisal of stressful situations (Tafet et al., 2003), and that CRH may be pivotal for this function. While increased CRH responses to acoustic startle have been reported within the extended amygdala (Davis et al., 1997), it is unclear whether the same effects would be present in the CeA. Results from the present study indicated that BALB/cByJ mice

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displayed significantly lower basal CRH levels within the CeA than C57BL/6ByJ mice, and that the changes of CRH expression in the CeA under acute stress situations, may be strain dependent. Although the acoustic startle responses did not differ between the two strains, greater neuroendocrine responses to the stressor were previously observed in the BALB/cByJ mice (Anisman et al., 2001). The increase of CRH content within the CeA following the acute stressor that was observed in BALB/cByJ mice in the current study, is consistent with the increased reactivity to stressors that is typically seen in this strain. Conversely, the reduction of CRH-ir in C57BL/6ByJ mice may have reflected adaptation of neuronal responses to the auditory stimulus or habituation with respect to the meaningfulness of the acoustic startle stressor.

It appears that there is a particular time course of stressor-induced CRH release in the amygdala. A significant increase of CRH mRNA expression was found 1 hour after acute restraint, but returned to baseline levels 3 hours following the stressor (Kalin et al., 1994). The CeA also showed a rapid stressor-induced increase of CRH mRNA (Hsu et al., 1998); however it is not known whether the expression is maintained for several hours, or rapidly declines back to baseline levels. If the rapid rise and fall was similar within the CeA, and also correlated to CRH content, then results from the present study may be inaccurate, given that CRH levels were only measured at 3 hours following stressor termination. Moreover, it is equally possible that the two mouse strains exhibit different time frames of CRH release within the CeA.

The available data concerning central amygdala CRH following chronic stressors are limited. It was shown that subordinate rats in a chronic psychosocial stressor study, showed increased CRH mRNA expression within the CeA (Albeck et al., 1997).

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Furthermore, in contrast to acute stressors, the increase of CRH following a chronic stressor treatment appears to be more sustained, being present even one day after a final stressor exposure (Albeck et al., 1997). Our results are inconsistent with these findings, as the chronic stressor regimen did not elicit any changes of CRH content within the CeA, even when measured 3 hours following stressor termination. It is possible that the nature of the stressors employed is differentially regulated by CRH within the CeA. While chronic psychosocial stressors used in previous studies may preferentially activate CRH within the CeA, chronic neurogenic and psychogenic stressors may involve CRH-independent mechanisms. Indeed, Merali et al (2003, 2004b) argued that independent stress pathways exist, and some stressors, such as those involving psychogenic insults, may be more effective in eliciting CeA CRH changes relative to stressors that are psychogenic in nature.

Glucocortiocoid responsiveness of amygdaloid CRH

CRH responses to stressors in the CeA appear to dependent on circulating GCs, and thus may be partially regulated by GC feedback in a feed-forward manner, unlike the feedback inhibition that is exerted on the PVN (Cook et al., 2002). Thus, CORT may regulate amygdaloid responses to stressors. Interestingly, the chronic repeated stressor treatment that was used in this study was found to potentiate the responsiveness of the amygdala to the novel stressor, and this was linked to the effects of CORT acting on type 2 GC receptors. Thus, it appears that contrary to the hypothalamus, the amygdala is not under the constraint of GC negative feedback, but rather GCs may exert a sensitization of amygdala neurons to subsequent stressors. This is consistent with the view that the

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amygdala is involved in anticipatory anxiety responses to stressors (Cook et al., 2002), as well as the suggestion that chronic stressors, by virtue of the high circulating GCs elicited, contribute to the development of anxiety related illnesses. From this perspective, the well-established differences in GC responses to stressors between BALB/cByJ and C57BL/6ByJ strains may have contributed to the differences seen with respect to CRH within the CeA.

Amygdaloid CRH-mediated behavioural anxiety

Examination of the behavioural data (as will be discussed later) clearly indicated that acute stressors provoked significant anxiety responses in BALB/cByJ mice, a finding consistent with the view that activation of CRH within the CeA is essential to promoting anxiety responses (Dunn & Berridge, 1990; Kalin et al., 1994). However, as already indicated, the CRH changes were modest, and did not reach statistical significance, and hence it ought to be considered that CRH release may not have been necessary for the behavioural expression of anxiety. Indeed, studies have shown that CRH antagonists applied to the amygdala do not necessarily abolish the anxiety response to several types of stressors, including a novel environment, elevated plus-maze and light/dark exploration (Bale et al., 2002; Griebel et al. 1998; Heinrichs et al., 1994; Okayama et al., 1999; Spina et al., 2000). However, CRH antagonists attenuated anxiety responses to stressors involving conditioned fear, shock-induced freezing, conditioned defeat and defensive withdrawal (Hikichi et al, 2000; Jasnow et al., 1999; Swiergiel et al., 1993). Together, these data suggest that anxiety-promoting CRH release in the amygdala is largely dependent on the nature of the stressor. Indeed, it will be recalled that Merali et al

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(2003, 2004b) argued that different neural circuits might be involved in mediating different stressor effects leading to anxiety reactions.

In the present investigation an acoustic startle stimulus was used to elicit a stress response, as this stressor has been shown to effectively increase amygdaloid CRH release (Merali et al., 2004b). Moreover, intracerebroventricular infusion of CRH enhanced the acoustic startle reflex in rats (CRH-enhanced startle), an effect that was blocked by electrolytic lesions to the amygdala (Liang et al., 1992). However, CRH infused directly into the amygdala failed to elicit such effects. It was suggested that CRH may bind directly to other structures such as the BNST, hippocampus, and the nucleus reticularis pontis caudalis, and direct excitatory connections between these areas and the amygdala could potentially contribute to the enhanced startle responses (Lee & Davis, 1997). In this regard, Lee and Davis (1997) showed that CRH in the CeA, in particular, may not be involved in the expression the acoustic startle reflex, but is more important in the expression of fear-potentiated startle. Lesions of the BNST, but not CeA or basolateral nucleus of the amygdala (BLA) were found to block CRH-enhanced startle. Conversely, lesions to the CeA and BLA, but not the BNST blocked fear-potentiated startle. These data indicate that CRH in the CeA may not be directly involved in the expression of the unconditioned acoustic startle response, however connections between the CeA and BNST may mediate these effects. In effect, these data raise the possibility that anxiety differences between the BALB/cByJ and C57BL/6ByJ strains may not be mediated by CRH variations within the CeA, but may involve brain regions that influence CeA functioning.

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It is possible that the stressor treatments employed in the present investigation may have preferentially activated CRH or CRH receptors within other regions of the amygdala such as the BLA. This region is strongly activated by stressors such as restraint, footshock and swim (Herman et al., 2003), and has been shown to influence HPA regulation, as well as the fear and anxiety responses associated with stressors. In fact, the anterior BLA has been shown to extensively innervate the CeA (Herman et al., 2003). While few CRH_1 receptors are present within the CeA, they are abundant in the BLA, and it has been suggested that sensory information entering the amygdala may preferentially provoke CRH changes within the BLA, and relay behavioural information to the CeA (De Souza et al., 1985; Herringa et al., 2004; Lee & Davis, 1997).

Hypothalamic CRH expression following acute and chronic stressors

The acute stressor used in the present experiment was expected to provoke an increase of CRH content within the ME, based on previous findings pointing to CRH as being the primary mediator of ACTH release under acute stressor conditions (Cook, 2004; Imaki et al., 2001; Makino et al., 1999; Makino et al., 1995; Pinnock & Herbert, 2001). However, no such changes were observed. It has been reported that among BALB/cByJ mice, a significant decrease of CRH levels occurred in the PVN within minutes following termination of a stressor, while C57BL/6ByJ mice exhibited the opposite effects, displaying a significant increase of CRH levels. The decrease of CRH in BALB/cByJ mice was accompanied by an increase of pituitary CRH levels, indicating that the reduction that was seen in the PVN was likely a result of the depletion of CRH stores within the area, owing to increased HPA activation (Anisman et al., 1998). These

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findings support the idea that BALB/cByJ mice exhibit greater HPA responses to stressors than the C57BL/6ByJ strain, and furthermore, parallel the opposing effects that were seen between the two strains with respect to CRH receptor changes in the PFC in response to the acute stressor. However, likely due to the lengthy period of time following stressor termination that the animals were sacrificed, we were not able to detect any CRH changes within the two strains of mice following the acute stressor.

Consistent with previous research (DeGoeij et al., 1991, 1992a; Hashimoto et al., 1988; Pinnock & Herbert, 2001), in the present investigation CRH content within the ME did not significantly change following chronic stressor treatment. However, other studies have revealed conflicting results. For example, 2 weeks of chronic variable stressor exposure reduced CRH concentrations within the ME by 24% (Stout et al., 2002). Furthermore, repeated immobilization induced a 75% increase in the number of CRH-positive neurons (DeGoeij et al., 1992b). These inconsistencies may stem from the variability in duration of the stressors, as well as the length of time following the stressor at which the animal was sacrificed. Furthermore, as previously discussed, analysis of CRH content within the PVN and ME is difficult because of its rapid turnover. Whether such factors, or the time at which the brain was taken following stressor exposure was responsible for the absence of an effect on CRH content, is presently uncertain.

Hypothalamic CRH and AVP co-localization following acute and chronic stressors

It has been reported that chronic stressors elicit an increase in the co-expression of CRH and AVP within the external zone of the median eminence. In this regard, it was expected, based on previous research, that an acute stressor would elicit an increase of

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CRH expression (Cook, 2004; Imaki et al., 2001; Makino et al., 1999; Makino et al., 1995; Pinnock & Herbert, 2001), while chronically stressed animals would exhibit a marked increase of CRH/AVP co-expression within the ME (Bartanusz et al., 1993; DeGoeij et al., 1991, 1992a, 1992b). Studies examining CRH/AVP co-expression within the ME and PVN, in fact, suggested that AVP may be an important mediator of the effects of chronic stressors in particular. Repeated immobilization, adrenalectomy, and chronic psychosocial stressors increased AVP content within CRH terminals of the external zone of the ME (Bartanusz et al., 1993; DeGoeij et al., 1991, 1992a, 1992b), as well as within parvocellular neurons of the PVN (Bartanusz et al., 1993; Makino et al., 1995). However, results from the present investigation failed to show such effects. Neither acute nor chronic stressors influenced CRH/AVP co-localization or CRH expression in the ME within either of the two mouse strains examined.

It has been suggested that parvocellular AVP may display a greater sensitivity to GC negative feedback than CRH (Ma & Aguilera, 1999a; Makino et al., 1995). Indeed, this perspective was supported by the finding that a transient suppression of CORT release, and the reduced ensuing hormone levels caused an increase of AVP within CRH terminals (Schmidt et al., 1997). Furthermore, AVP receptor expression and binding also increased following chronic but not acute stressor treatment, and this was correlated with the responsiveness of ACTH following the treatment (Aguilera, 1994; Aguilera et al., 1994; Rabadan-Diehl et al., 1995). As GC negative feedback may be disrupted following chronic stressors, it would be expected that AVP levels would rise. In effect, the shift of importance from CRH to AVP with respect to HPA activation following repeated stressors may be mediated by the greater sensitivity of AVP to GC negative feedback, in

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addition to other GC-independent mechanisms (Makino et al., 1995). Based on these findings, it appears that AVP plays a critical role in the maintenance of HPA reactivity, despite the high circulating levels of GCs during exposure to chronic stressors.

Recent studies that assessed the effects of AVP1b receptor antagonists in behavioural models of anxiety and depression have also alluded to an important role for AVP in chronic stressor situations. In particular, treatment with an AVP1b receptor antagonist (SSR149415) significantly reversed the reduction of cell proliferation and prevented the reduction of granule cell neurogenesis that ordinarily occurred following a chronic mild stressor regimen (Alonso et al., 2004). Moreover, the AVP1b antagonist promoted an anxiolytic-like effect in response to stressors, and attenuated the behavioural symptoms of anxiety and depression produced by a chronic mild stressor regimen (Griebel et al. 2002). These studies point to the importance of AVP in the development of affective and anxiety disorders, and thus, its probable role in HPA functioning.

Caveats concerning CRH-AVP alterations within the ME

Given the array of studies that have confirmed the role of AVP in mediating the effects of chronic stressors, a number of factors that may have affected our results ought to be considered. First, turnover of CRH and AVP within the ME is very rapid, and therefore determination of their content within the ME in response to a stressor would be inaccurate, unless axonal transport was blocked by using colchicine, for example (Anisman et al., 1998; Berkenbosch & Tilders, 1988; Moldow et al., 1987). In a chronic psychosocial stressor paradigm (DeGoeij et al., 1992a), colony-housed subordinate rats displayed markedly increased AVP-ir within the external zone of the ME, which was

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accompanied by elevated resting CORT levels. Interestingly, the increase of AVP immunostaining within the ME was not associated with an increase in the secretion of the peptide, as measured after blockade of axonal transport. This might signify that while AVP stores within the ME may increase following a chronic stressor treatment, the AVP was not released, and thus may not have necessarily contributed to the increase of basal CORT levels. While adrenalectomized rats also showed increases of AVP stores within the external zone of the ME, blockade of axonal transport caused a progressive depletion, indicating that the increased AVP reflected an increase of AVP secretion from the ME (DeGoeij et al., 1993). Together, these studies make it clear that that CRH-ir and AVP-ir within the external zone of the ME are only indicative of peptide stores, and do not necessarily reflect secretion of the peptides. In addition, owing to the high turnover of these peptides, determination of CRH and AVP in the ME would benefit from analyses within minutes following a brief stressor, in contrast to the lengthy duration of the final stressor that was used (30 minutes of acoustic startle), as well as the 3 hour wait following stressor termination in the present investigation.

It is essential to distinguish between AVP-positive CRH terminals within the external zone of the ME, from those that are AVP-deficient. It was shown that an acute immobilization stressor caused a marked depletion of vesicles within AVP-positive CRH terminals of the ME, but CRH terminals that were AVP-deficient remained unaltered by the stressor exposure (DeGoeij et al., 1991; Whitnall, 1989). The differential activation of AVP-positive and AVP-deficient CRH terminals indicates that the two peptides may be independently regulated. In the present experiment, the total CRH-ir within the external zone of the ME was assessed, and did not distinguish between AVP-positive and AVP-

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deficient terminals. In this respect, the CRH content within AVP-positive terminals may actually be affected by acute and chronic stressor exposure, but by measuring the total ME CRH-ir, such changes may be obscured.

We cannot exclude the possibility that the time courses of CRH and AVP expression following a stressor are different, which may also have contributed to the failure to find any significant changes of CRH or AVP content within the ME. Consistent with this, an acute swim stressor induced a marked increase of CRH hnRNA within the parvocellular PVN, 10 minutes following the stressor, but returned to basal levels 2 hours later (Jiang et al., 2004). In contrast, AVP hnRNA only increased 2 hours following the stressor. Furthermore, these findings indicate that the mechanisms of gene transcription for CRH and AVP are different, and reveal differences in their relative importance following acute and chronic stressors. While CRH may be in immediate demand, being more important following acute stressors, the delayed activation of AVP suggests its relevance in response to chronic stressor exposure (Jiang et al., 2004). These findings once again raise the possibility that by measuring CRH and AVP levels at only one time point following the stressors, we may be missing any changes that may have occurred before or after that time. As indicated earlier, in future experiments, it would be preferable to measure CRH and AVP levels *in vivo* (by using microdialysis), in order to determine dynamic changes of peptide expression.

It is possible that limitations in the quantitative analytic method that was used in the present experiment contributed to the failure to detect differences of CRH/AVP co-expression within the ME following the stressor treatments. Analysis for co-localization of CRH and AVP was based on thresholding the deconvolved images to retain only high-

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intensity specific labeling of each peptide signal, based on a method derived from Hutcheon et al. (2000). Thus, only the signals within the top 5% of each antibody staining were used to determine the amount of CRH/AVP co-localization. While this level of thresholding has been shown to be physiologically relevant for analysis of receptors (Hutcheon et al., 2000), it may have been too conservative for analysis of peptide levels at neuronal release sites, thus possibly concealing any differences between the experimental groups. It would be necessary to repeat the analysis using the top 10% as the thresholding level to confirm this.

In addition to measurement factors, it needs to be considered that certain characteristics of the stressor treatments themselves may have precluded detection of CRH-AVP alterations. However, it seems unlikely that the length or the nature of the chronic stressor regimen employed was insufficient to elicit changes of CRH and AVP expression. Previous studies have used much shorter periods (e.g. 2-3 weeks) (DeGoeij et al., 1992a; Stout et al., 2002), as well as homotypic stressor treatments (i.e. same stressor applied repeatedly) (DeGoeij et al., 1991, 1992b, 1993; Makino et al., 1995) and still observed marked changes of HPA functioning. Further, the extended period of chronic stressors used in the present experiment, in addition to the unpredictability and variety of stressors, has been shown to provoke pronounced disturbances of behavioural, neurochemical and neuroendocrine functioning (Tannenbaum et al., 2002; Tannenbaum & Anisman, 2003) and thus, the stressor parameters employed were likely appropriate to detect variations of peptidergic responses as well.

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Behavioural responses to acute and chronic stressor treatment

Consistent with previous findings, results from the open-field emergence test indicated that BALB/cByJ mice displayed increased symptoms of anxiety relative to mice of the C57BL/6ByJ strain (Anisman et al., 2001). BALB/cByJ mice had higher latencies to leave the dark box, spent less time exploring in the open-field, and displayed a greater number of stretch attends than the C57BL/6ByJ mice. These findings are consistent with previous research that has shown diminished stressor reactivity in C57Bl/6ByJ mice, in not only the open-field emergence test, but also several other measures of anxiety (Anisman et al., 2001; Tannenbaum & Anisman, 2003). However, the chronic stressor treatment did not appear to affect any of these behaviours. Based on previous research employing the same type of chronic stressor regimen within these mouse strains, it was expected that anxiety responses would become more pronounced following the chronic stressor treatment, particularly within BALB/cByJ mice (Tannenbaum & Anisman, 2003). The fact that the chronic stressor did not augment these responses was not unexpected in the resilient C57BL/6ByJ strain, but the absence of such an effect in BALB/cByJ mice was clearly unexpected. At this juncture it is difficult to discern what factors might have been responsible for the lack of augmented anxiety in the latter strain, but the small number of subjects, coupled with the high variance may have contributed to this outcome.

In the forced swim test, non-stressed BALB/cByJ mice showed only marginal decreases in the amount of active swimming that was displayed over trials, whereas non-stressed C57BL/6ByJ mice displayed a precipitous decline of active swimming over the trials. The chronic stressor regimen appeared to have a different impact on the two mouse

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strains. Among BALB/cByJ mice, the chronic stressor produced a significant decline of active swimming relative to non-stressed BALB/cByJ mice. This finding is consistent a previous report using a similar chronic stressor regimen in CD-1 mice (Tannenbaum et al., 2002). In contrast, chronically stressed C57BL/6ByJ mice exhibited a marginally lower amount of active swimming on the initial trial, but increased this behaviour over trials.

The reduction of active swimming in this test has commonly been interpreted as a sign of depression, as these effects were attenuated by antidepressant treatment (Cryan et al., 2005; Porsolt, 2000). The present findings are consistent with this perspective, with the reduction of active swimming in BALB/cByJ mice following the chronic stressor treatment possibly reflecting their increased vulnerability to depressive-like symptoms. However, this interpretation may not be relevant when examining different strains of mice. The reduced swimming seen in C57BL/6ByJ control animals is unlikely a sign of depression, as these mice have consistently been shown to be more resilient to the effects of stressors relative to BALB/cByJ mice (Anisman et al., 2001; Tannenbaum & Anisman, 2003). Moreover, the increased swimming seen in this strain following the chronic stressor treatment may have reflected increased anxiety rather than exacerbation of behavioural depression (Prince et al., 1986; Prince & Anisman, 1984). Provisionally, it seems that a chronic stressor regimen may have activated different processes in the two strains, leading to the diametrically opposed behavioural styles, possibly reflecting different affective or arousal states.

Commensurate with previous findings, behavioural responses to the final acoustic startle stressor did not differ in BALB/cByJ and C57BL/6ByJ mice (Anisman et al.,

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2001). Indeed, it was for this reason that the acoustic startle was chosen as the final stressor. That is, any differences in HPA functioning or CRH changes within limbic regions could not be attributed to behavioural differences elicited by the immediate antecedent treatment. However, startle reflex responses did not differ between acutely and chronically stressed mice, possibly contributing to the absence of any differences of CRH/AVP co-localization in the ME, and CRH expression in the CeA between these groups.

Conclusions

The acute stressor treatment clearly provoked strain-specific variations of CRH receptors within the PFC as well as CRH peptidergic variations within the CeA. The opposing peptidergic outcomes in these strains paralleled reports of their differential stressor reactivity. The expression patterns of PFC CRH receptors following an acute stressor are consistent with the notion that the PFC exerts inhibitory feedback over HPA activity, and that this effect may be mediated, in part, by CRH receptors, although further research is required to confirm this presumption. The two CRH receptor subtypes (CRH_1 and CRH_2) have been shown to exert markedly different effects on behaviour and HPA activity. Whether these receptor subtypes differ in the two strains, and whether these contribute differentially to the strain-specific anxiety, remains to be determined.

The changes of CRH content within the CeA that were observed following an acute stressor, are consistent with the view that CRH in this region may be related to anxiety. In general, however, the chronic stressor treatment did not exacerbate the effects that were observed in either the PFC or CeA. For the moment, however, it is not possible

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to say whether the absence of a chronic stressor effect was due to the static method of measuring dynamic CRH variations. Ultimately, it will be necessary to assess these processes through in vivo measurements over time following a stressor experience.

Finally, although significant changes of CRH or AVP expression within the ME were not detected in the present investigation, it is uncertain whether this was a result of the particular stressors used or the specific method of measurement employed. While the physiological significance of having two ACTH secretagogues (CRH and AVP) is yet to be fully elucidated, having a dual regulatory system may aid in responding to a diverse range of stressors (Jiang et al., 2004). By understanding how these peptides are regulated following acute and chronic stressors, and particularly within the BALB/cByJ and C57BL/6ByJ strains, we can further consolidate an animal model for depression and anxiety-related disorders, which can be useful for research into the treatment of these illnesses.

With the increasing evidence that CRH and AVP are important mediators of the effects of stressors, recent research has focused on CRH and AVP receptor antagonists as potential novel therapeutic agents in the treatment of stressor-related illnesses (Ducottet et al., 2003; Griebel et al., 2002). The use of the BALB/cByJ and C57BL/6ByJ strains to illustrate the effects of these therapeutics may be a useful tool in understanding how these treatments may affect different patient groups or individuals differentially reactive to stressors.

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