NMDA RECEPTORS AND PCREB - THEIR ROLE IN BRAIN AND BEHAVIORAL CHANGES AFTER STRESS

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by

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Abstract

In this dissertation, we employed the ecologically valid predator stress model to assess the effects of stress on brain and behavior. A five minute unprotected exposure of a rat to a cat induces long-lasting changes in anxiety-like behaviors in the rat (Adamec & Shallow, 1993; Adamec et al., 2001; Cohen et al., 2004). Using this model, we addressed four main questions. First, are the various manifestations of the anxiogenic effects of predator stress *N*-methyl-*D*-aspartate (NMDA) receptor-dependent? Second, are the neuroplastic changes that occur after predator stress NMDA receptor-dependent? Third, do neuroplastic changes occur after exposure to other, milder stressors, such as the elevated plus maze (EPM)? Finally, is the neuroplastic response to a mild stressor enhanced in animals that have been previously stressed?

In Chapter 2, we assessed the effects of a NMDA receptor antagonist [(3-(2-carboxypiperazin4-yl)propyl-l-phosphonic acid) - CPP] on anxiety-like behavior produced by predator stress. An affect test battery including hole board, elevated plus maze (EPM), light/dark box, social interaction, social avoidance, and response to acoustic startle was employed to assess the behavioral response to stress. Doses of 1-10 mg/kg of CPP administered ip 30 min prior to predator stress blocked most anxiety-like behaviors measured eight and nine days after stress. CPP blocked the predator stress-induced reduction in open arm exploration and risk assessment in the EPM, blocked the predator stress-induced decrease in entries into the lighted arm of the light/dark box, and blocked the predator stress-induced delay in habituation of the acoustic startle response. Behaviors in which the effects of predator stress were not blocked by CPP included

reduction in unprotected head dips in the EPM and reduced social interaction. In addition, predator stress was without effect on social avoidance as measured with the Haller test. Taken together, these findings add to a body of evidence showing that a syndrome of behavioral changes follows predator stress. Components of this syndrome of behavioral changes likely depend on changes in separable neural substrates initiated by NMDA receptors as well as by other neurochemical means.

Since phosphorylation of cyclic AMP response element binding protein (CREB) is regulated by NMDA receptors and pCREB-like immunoreactivity (lir) is increased after predator stress, we examined the effects of CPP on predator stress-induced changes in pCREB-lir in Chapter 3. pCREB-lir was assessed using immunocytochemistry in brain areas implicated in fearful and anxious behavior including the amygdala, periaqueductal gray (PAG), bed nucleus of the stria terminalis (BNST), anterior cingulate cortex (ACC), and dorsal medial hypothalamus (DMH). Results showed that CPP blocked the predator stress-induced increase in pCREB-lir in the right lateral column of the PAG, blocked the predator stress-induced increase in pCREB-lir in several amygdala nuclei, and reversed the predator stress-induced suppression of pCREB-lir in the BNST. Importantly, at least in the amygdala and PAG, the pattern of pCREB-lir was hemisphere- and anterior-posterior (AP) plane-dependent. Our results suggest that several amygdala nuclei, the PAG, and the BNST, where predator stress changes pCREB-lir in a NMDA receptor-dependent manner, are candidate areas of neuroplastic change contributing to lasting changes in anxiety-like behavior. However, like predator

stress-induced changes in anxiety-like behavior, not all stress-induced changes in pCREB-lir were NMDA receptor-dependent.

In Chapter 4, we examined pCREB changes in response to other stressors, such as the EPM, in brain areas implicated in fear and anxiety. In addition, we investigated the effects of prior traumatic stress on pCREB-lir in animals exposed to the EPM. In particular, pCREB-lir was examined after exposure to the EPM in rats that had been exposed to a cat seven days earlier and naïve (handled) controls. Brain areas investigated for both experiments included the amygdala, PAG, and BNST, which are all areas that show NMDA receptor-dependent pCREB-lir changes after predator stress. Results showed that there were no pCREB-lir differences between naïve control rats and rats exposed to the EPM only. However, exposure to the EPM in predator stressed rats elevated pCREB-lir in the right lateral column of the PAG and bilaterally in the dorsal column of the PAG. Findings suggest mechanisms associated with neuroplasticity may be further engaged by relatively mild stresses in animals with a history of severe stress exposure.

Taken together, these results suggest that most changes in anxiety-like behavior following predator stress are NMDA receptor-dependent. In addition, changes in pCREB-lir in several amygdala nuclei, the right lateral column of the PAG and the BNST may mediate the predator stress-induced increases in anxiety-like behavior. Furthermore, mechanisms associated with neuroplasticity may be further activated by relatively mild stresses in animals with a history of severe stress exposure.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACC	Anterior cingulate cortex
ACo	Anterior cortical amygdala
ALB	Anxiety-like behavior
ANOVA	Analysis of variance
AP	Anterior posterior
BAOT	Bed nucleus accessory olfactory tract
BLa	Basolateral amygdala
BLv	Basolateral ventral amygdala
BM	Basomedial amygdala
BNST	Bed nucleus of the stria terminalis
cAMP	Cyclic adenosine 3',5'-monophosphate
Ce	Central amygdala
CPP	(3-(2- carboxypiperazin4-yl)propyl-l-phosphonic acid)
CREB	cAMP-response element binding protein
CRF	Corticotrophin Releasing Factor
DMH	Dorsal medial hypothalamus
dPAG	Dorsal periaqueductal gray
EPM	Elevated plus maze
HPA	Hypothalamic–pituitary–adrenal
ICC	Immunocytochemistry
ip	intraperitoneal
La	Lateral amygdala
lir	Like-immunoreactivity
lPAG	Lateral periaqueductal gray
LTP	Long-term potentiation
Me	Medial amygdala
NGS	Normal Goat Serum
NMDA	*N*-methyl-*D*-aspartate
OD	Optical Density
PAG	Periaqueductal gray
PBS	Phosphate buffered saline
PCo	Posterior cortical amygdala
pCREB	phosphorylated cAMP-response element binding protein
PKA	cAMP-dependent protein kinase A
PTSD	Posttraumatic stress disorder
VAB	Ventral angular bundle

List of Appendices

Chapter 1: Introduction

The effects of traumatic experiences have been documented for centuries. For instance, Da Costa (1871), an American physician treating casualties of the American Civil War (1861-1865), described increased arousal, irritability, and elevated heart rate in soldiers exposed to combat. This group of symptoms became known as "Da Costa's Syndrome," or "Soldiers Irritable Heart", and was a result of exposure to the stress of combat (Trimble, 1981). During the First World War, Freud (1917) theorized that war trauma "presents the mind with an increase of stimulus too powerful to be dealt with or worked off in the normal way, and this must result in permanent disturbances (p. 275)." The following is an autobiographical description of a British lieutenant who was hospitalized in Great Britain after being trapped behind enemy lines in France, recorded by Mott (1919).

> "At the present time I am subject to dreams in which I hear these shells bursting and whistling through the air. I continually see my sergeant, both alive and dead, and also my attempts to return are vividly pictured. I sometimes have in my dreams that feeling of intense hunger and thirst which I had in the village. When I awaken I feel as though all the strength has left me and I am in a cold sweat. For a time after awaking I fail to recognize where I am, and the surroundings take on the form of the ruins in which I remained hidden for so long. Sometimes I do not think that I am thoroughly awakened, as I seem to doze off, and there are conflicting ideas that I am in the hospital, and again that I am in France. During the day, if I sit doing nothing in particular and find myself dozing, my mind seems to immediately begin to fly back to France (p.126-127)."

During the Second World War, mental health practitioners evaluated and treated many psychiatric casualties (Lewis, 1942). Compelled by the prevalence of war-related psychiatric morbidity, the American Psychiatric Association's (APA) Committee on

Nomenclature and Statistics included the classification *gross stress reaction* in the Diagnostic and Statistical Manual of Mental Disorders (DSM-I) of 1952 (APA, 1952). Since then, much research has been done on the behavioral and psychiatric response to severe stressors and in 1980 the DSM-III set the diagnostic criteria for posttraumatic stress disorder (PTSD) (APA, 1980). PTSD can be a debilitating disorder characterized by severe anxiety, nightmares, agitation and often depression. The traumatic event is persistently re-experienced as distressing recollections, dreams, sudden feeling as if the traumatic event were recurring (illusions or hallucinations) or intense psychological distress when exposed to events that symbolize or resemble an aspect of the traumatic event.

Anxiety associated with traumatic stress is an important area of research as 61% of males and 51% of females in North America experience some form of traumatic stress in their lifetime (Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995). More importantly, of those percentages, approximately 15 % may develop PTSD (Kessler, McGonagle, Zhao, Nelson, Hughes, Eshleman, Wittchen, Kendler, 1994; Kessler *et al.*, 1995). Furthermore, prior exposure to a stressor can increase the likelihood of developing psychopathology (i.e., PTSD) following subsequent traumatic stress (Bremner, Southwick, Johnson, Yehuda, & Charney, 1993; Solomon and Davidson, 1997). More recently, with the attacks on the World Trade Center in New York and increased global terrorism, the need for more research into the effects of stress on brain function is even more important. In fact, recent studies estimate in excess of 500,000 cases of PTSD have emerged in the New York City area as a result of the occurrences on

September 11th, 2001 (Galea, Resnick, Ahern, Gold, Bucuvalas, Kilpatrick, Stuber, & Vlahov, 2002; Schlenger, Caddell, Ebert, Jordan, Rourke, Wilson, Thalji, Dennis, Fairbank, & Kulka, 2002). There is no cure for this disorder; thus, the study of the effects of stress on brain and behavior is particularly important.

This introduction will begin with an examination of the criteria and symptoms associated with PTSD. Several animal models used to understand PTSD will be described, including the model used in the present studies. I will conclude with a discussion of relevant behavioral, physiological, and molecular changes that occur in rodents after predator stress, which will lead into the justification for the three studies.

1.1 Diagnostic Criteria for PTSD

There are six criteria outlined in the DSM-IV and of those, at least two must be present for a diagnosis of PTSD (APA, 1994). The criteria include insomnia, an intensified symptom profile during recall of the initiating event; avoidance of events associated with the trauma, guilt associated with the event, a general difficulty in concentrating or remembering, and an exaggerated startle response. If the symptoms are present for less than three months, the individual is classified as having acute PTSD. The disease is considered chronic if the symptoms persist for over three months. Finally, if at least six months have passed between the traumatic event and the onset of symptoms, the individual is considered to have PTSD with delayed onset.

1.2 HPA Abnormalities in PTSD

Exposure to trauma activates the hypothalamic–pituitary–adrenal (HPA) axis. As a result, PTSD patients have abnormal stress hormone release (Van der Kolk, 1994). For instance, many studies, especially those examining women, have found evidence of HPA axis hyperactivity, particularly after childhood abuse (Heim, Newport, Wagner, Wilcox, Miller, Nemeroff, 2002; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Maes, Lin, Bonaccorso, van Hunsel, Van Gastel, Delmeire, Biondi, Bosmans, Kenis, & Scharpe, 1998; Pitman & Orr, 1990). In addition, elevated basal cortisol levels have been reported in women who suffer from PTSD due to partner violence (Pico-Alfonso, Garcia-Linares, Celda-Navarro, Herbert, & Martinez, 2004). However, studies by Yehuda and others in male combat veterans and elderly Holocaust survivors with PTSD have demonstrated "hypocortisolism" and enhanced negative feedback to low-dose dexamethasone (Boscarino, 1996; Yehuda, 2002; Yehuda, Bierer, Schmeidler, Aferiat, Breslau, & Dolan, 2000; Yehuda, Southwick, Krystal, Bremner, Charney, & Mason, 1993). Recently, Young and Tolman (2004) examined salivary cortisol in a community sample of low-socioeconomic-status women with high exposure rates to trauma in both childhood and adulthood and found normal cortisol in women with current and lifetime PTSD. Similarly, a large community study found neither exposure to trauma nor PTSD alone was associated with alterations in salivary cortisol; however, elevated cortisol was found in PTSD co-morbid with lifetime major depressive disorder (Young and Breslau, 2004). Due to the inconsistencies in the results, it is possible that the stress response is related to the specific stressor and the victim's characteristics making generalizations difficult.

1.3 Changes in Acoustic Startle

Changes in startle amplitude and habituation of startle have been found in PTSD patients. Butler, Braff, Rauch, Jenkins, Sprock, and Geyer (1990) tested Vietnam veterans with PTSD and non-PTSD veterans for acoustic startle response using eyeblink electromyogram amplitudes. Results showed that PTSD patients exhibited higher eyeblink amplitudes than non-PTSD veterans. In addition, Kolb (1987) found differences in PTSD and controls in both blood pressure and galvanic skin response when presented with combat sounds, whereby PTSD veterans showed a greater response to the sounds than controls.

Habituation to acoustic startle is also changed in PTSD patients. Orr, Lasko, Shalev, and Pitman (1995) compared the startle responses of Vietnam veterans with and without PTSD. They found that veterans with PTSD exhibited larger heart rate and eyeblink responses. As well, skin conductance response magnitude declined more slowly across trials for veterans with PTSD than for non-PTSD veterans.

1.4 Brain Areas Associated with Fear and Anxiety in Humans

Vietnam veterans suffering from PTSD show an increase in Positron Emission Tomography (PET) activation of the right amygdala in response to stimuli that remind them of their combat trauma (Shin, McNally, Kosslyn, Thompson, Rauch, Aplert, Metzger, Lasko, Orr, & Pitman, 1997). This is consistent with Rauch and colleagues who found increased blood flow in right-sided limbic, paralimbic, and visual areas following traumatic reminders (Rauch, van der Kolk, Fisler, Alpert, Orr, Savage,

Fischman, Jenike, & Pitman, 1996). Furthermore, electrical stimulation of the amygdala has been shown to induce physiologic and emotional signs of anxiety and fear and can cause recollections of emotionally salient life experiences from remote memory (Drevets, Videen, Price, Preskorn, Carmichael, & Raichle, 1992; Gloor, Olivier, Quesney, Andermann, & Sorowitz, 1982). In addition to the amygdala, Nashold, Wilson, and Slaughter (1969) reported feelings of intense fear or panic during stimulation of the periaqueductal gray (PAG). Patients became apprehensive and would not allow further stimulation of the area. The anterior cingulate cortex (ACC) has also been implicated in emotional processing (Garavan, Pankiewicz, Bloom, Cho, Sperry, Ross, Salmeron, Risinger, Kelley, & Stein, 2000), as lesions in this area have produced a wide variety of symptoms, including apathy, inattention, autonomic dysregulation, emotional instability and akinetic mutism (Bush, Luu, & Posner, 2000; Devinsky, Morrell, & Vogt, 1995). Moreover, dysfunction and volumetric reductions in the ACC have been associated with PTSD (Rauch, Shin, Segal, Pitman, Carson, McMullin, Whalen, Makris, 2003; Shin, Whalen, Pitman, Bush, Macklin, Lasko, Orr, McInerney, & Rauch, 2001). These brain areas, as well as others, have been associated with fear and anxiety in animal models of PTSD.

1.5 Animal Models of PTSD

Animal models are useful because they allow the opportunity to simulate a severe stressor in a controlled fashion; the affective disorder can be studied as it develops; and pharmacological and other treatments may be difficult to test in humans but can be easily

evaluated in animals. A good animal model of PTSD must produce long-lasting anxiety, show fluctuation in stress hormones, show changes in brain areas relevant to brain areas associated with PTSD, and affect the startle response both by enhancing it and delaying its habituation. Conditioned fear paradigms, exposure to pharmacological stressors and more recently, predator stress, are all models used to explore the neurobiology of fearful events.

1.5.1 Classical Fear Conditioning

Classical fear conditioning links the trauma with the symptoms of PTSD. It has been suggested that the feelings of fear and extreme anxiety the victim experiences at the time of the trauma can become conditioned to a variety of stimuli present at the time of the trauma (Blair, Schafe, Bauer, Rodrigues, & LeDoux, 2001; Kolb & Multalipassi, 1982; Maren, De Oca, & Fanselow, 1994; Rogan, Stäubli, & LeDoux, 1997; Schafe, Nader, Blair, & LeDoux, 2001). This can be modeled in animals whereby a neutral stimulus (e.g., tone) can elicit defensive behaviors (e.g., freezing, enhanced startle) if the tone was previously paired with an aversive stimulus (e.g., shock).

Much work has been done on the neural changes involved in fear conditioning. In particular, neuroplasticity and long term potentiation (LTP) in amygdala circuitry contribute to both conditioning and extinction of fear (Blair et al., 2001; Myers & Davis, 2002; Nader, Schafe, & LeDoux, 2000; Schafe, Atkins, Swank, Bauer, Sweatt, & LeDoux, 2000; Schafe et al., 2001; Schafe & LeDoux, 2000). LTP can be described as a long-lasting enhancement in synaptic efficacy that has the properties expected of a

memory mechanism (i.e., long-lasting, associativity, and reversibility) (Izquieredo, 1994). Furthermore, conditioning and extinction of fear appear to be *N*-methyl-*D*-Aspartate (NMDA) receptor-dependent (Baker & Azorlosa, 1996; Campeau, Miserendino, & Davis, 1992; Davis, 2002; Decola, Kim, & Fanselow, 1991; Fanselow, Kim, & Yipp, 1992; Kim, Decola, Landeira-Fernandez & Fanselow, 1991; Lee, Choi, Brown, & Kim, 2001; Lee & Kim, 1998; Stiedl, Birkenfeld, Palve, Spiess, 2000). In the amygdala, most forms of neuroplasticity associated with the acquisition and extinction of fear conditioning are also NMDA receptor-dependent (Davis, 2002; Goosens & Maren, 2002; Lee, Lee, & Choi, 2002; Royer & Pare, 2002; Tsvetkov, Carlezon, Benes, Kandel, Bolshakov, 2002).

There is clinical interest in using this classical fear conditioning model to gain insights into the mechanisms of onset of PTSD as a guide to post-stressor prophylactic intervention in humans (Pitman, Sanders, Zusman, Healy, Cheema, Lasko, Cahill, & Orr, 2002) and to understand and improve the therapeutic outcome of exposure therapies (Myers & Davis, 2002). For example, the β-adrenergic blocker propranolol administered pre- (Quirarte, Roozendaal, & McGaugh, 1997) or post- (Cahill, Pham, & Setlow, 2000) conditioning abolishes the enhancement of memory produced by emotional arousal in rats and humans. As a result, propranolol has now been used clinically, with success, after trauma to prevent or reduce PTSD symptoms (Pitman et al., 2002; Vaiva, Ducrocq, Jezequel, Averland, Lestavel, Brunet, & Marmar, 2003).

Despite the merits of fear conditioning as a model of PTSD, there are several concerns. For instance, it has been argued that conditioning does not account for the

sensitized fearfulness manifested as generalized anxiety, which is also a key feature of PTSD (Pitman, 1997). In particular, it cannot account for the hyper-arousal, manifested as an exaggerated startle response, seen in human PTSD patients. Furthermore, it cannot explain patients with delay of onset PTSD (Pitman, Orr & Shalev, 1993; Shalev, 1993).

1.5.2 Pharmacological Stressor in Felines

Both behavioral and physiological research on stress in felines has relied on the use of pharmacological stressors such as benzodiazepine inverse agonists. The benzodiazepines have been the drugs of choice in the treatment of anxiety and anxiety-related disorders over the past several decades. Benzodiazepine receptor agonists tend to be anxiolytic, such as Valium. On the other hand, some inverse benzodiazepine agonists (*e.g.* N-methyl-beta carboline, 3 carboximide or FG-7142) cause intense anxiety in humans and mimic many of the brain and behavioral changes associated with severely stressful experiences (Dorrow, Horowski, Paschelke, Amin & Braestrup, 1983). Antagonists, in general, tend to reverse the effects of both agonists and inverse agonists without altering mood or behavior (File & Baldwin, 1989).

Effects of FG-7142 are blocked by anxiolytics in humans (Dorrow, Horowski, Paschelke, Amin, & Braestrup, 1983) and benzodiazepine antagonists, such as Flumazenil, in animals (Ongini, 1983). Adamec (1991) has shown that a single dose of FG-7142 produces long-lasting emotional disturbances (increased defensiveness) in cats. Specifically, increased defensiveness is seen in the presence of a rat and in response to

recorded howls of a threatening cat. The behaviors measured are considered to be an index of cat anxiety.

In the cat, FG-7142 produces equally long lasting-changes in limbic physiology, which closely correlate with the behavioral changes. In particular, FG-7142 induces lasting increases in defensive responsiveness that is closely associated with LTP of excitatory transmission from the amygdala to the lateral PAG (lPAG) (Adamec, 1997; Adamec, 1998a; Adamec, Kent, Anisman, Shallow, & Merali, 1998). Initially, LTP appears in both hemispheres, however, LTP is longer-lived in the right amygdalo-PAG pathway. Furthermore, right amygdalo-PAG potentiation lasts as long as the behavioral changes. Reversal of right amygdalo-PAG LTP with low frequency stimulation (LFS) selectively reduces defensive behavior in the cat (Adamec, 1999). Importantly, LFS does not affect predatory behavior suggesting specificity to defensive behavior of LTP in this pathway.

1.5.2.1 NMDA Receptor Dependence of Physiological and Behavioral Changes Produced by FG-7142

Some, but not all types of LTP depend on the activation of NMDA receptors (Adamec, 1997; Adamec, 1998a, Adamec et al., 1998; Adamec, Burton, Shallow & Budgell, 1999a; Maren, 1996; Rogan & LeDoux, 1995). The NMDA receptor blocker, AP7, administered before FG-7142 prevents both long-lasting increases in excitatory transmission in amygdala efferents and the increase in defensive behaviors in the cat (Adamec, 1998a; Adamec, 1998b). Thus, Adamec (1998a) has suggested that an NMDA

receptor-dependent form of LTP in amygdala efferents may underlie the behavioral changes produced by FG-7142. This finding is consistent with the effects of a severe stress (e.g., predator stress) on rodents.

1.5.3 Predator Stress

Predator stress is both fear provoking and stressful (Adamec et al., 1998; Blanchard, Nikulina, Sakai, McKittrick, McEwen, & Blanchard, 1998; Dielenberg, Carrive, & McGregor, 2001a; McGregor, Schrama, Ambermoon, & Dielenberg, 2002). In particular, cat exposure elicits immediate behavioral responses and induces long-lasting changes in defensive and emotional behaviors in rats. For example, rats housed in an artificial burrow system respond to the presentation of a cat with immediate fleeing into the burrow followed by immobility (Blanchard et al., 1998). After exposure to the cat, behavior of the rats changes significantly over the next 24 hours. For instance, rats withdraw to more distant burrow areas and their risk assessment behaviors, such as head extension out of the tunnel into the surface area, increase. In addition, non-defensive behaviors such as mating and feeding are suppressed. Zangrossi and File (1992) assessed the behavior of rats exposed to a cloth previously rubbed against the fur of a cat. Thirty minutes after exposure, rats displayed more anxiety-like behaviors (ALB) in the elevated plus maze (EPM). Similarly, rats exposed to a collar worn by a cat in a testing cage hide more and show more risk assessment when placed back in the testing cage 24 hours later (Dielenberg, Arnold, & McGregor, 1999; McGregor et al., 2002).

Similar to the conditioning studies, predator stress-induced changes in ALB involve the amygdala. Large lesions of the amygdala in rats block defensive behavior in response to a cat (Blanchard & Blanchard, 1972; Fox & Sorenson, 1994). In addition, smaller lesions or chemical inactivation of specific amygdala nuclei, such as the medial nucleus (Me) and the associated bed nucleus of the stria terminalis (BNST), reduce defensive freezing to cat or fox odor (Fendt, Endres, & Apfelbach, 2003; Li CI, Maglinao, & Takahashi, 2004). However, lesions or inactivation of other amygdala nuclei, including the basal (B), lateral (La), and central (Ce), have little effect on freezing to predator odors (Fendt et al., 2003; Li CI et al., 2004; Rosen, 2004; Wallace & Rosen, 2001).

1.5.4 Predator Stress Model used in the Current Studies

The predator stress model used in the present set of studies was developed in the Adamec laboratory (Adamec & Shallow, 1993). It involves the unprotected exposure of a rat to a cat for five minutes. The rat is placed in a room with a cat to allow the cat to approach and gently paw the rat. Predator stress lastingly increases ALB (Adamec & Shallow, 1993; Adamec, 1997), potentiates transmission in amygdala afferent and efferent pathways (Adamec, Blundell & Collins, 2001; Adamec, Blundell, & Burton, 2003) and increases phosphorylated cyclic AMP response element binding protein (pCREB) in several amygdala nuclei and the right lPAG (Adamec et al., 2003; Adamec, Blundell & Burton, *in press*).

1.5.5 Predator Stress as a Model of PTSD

It has been argued that predator stress models aspects of PTSD for several reasons. First, although the rat is not physically injured, cat exposure creates a species relevant life threatening experience that causes behavioral and neuroendocrine changes (Adamec et al., 1998). Thus, predator stress has a high degree of ecological validity in that it mimics a brief, intense life-threatening encounter with lasting affective consequences. Second, path analysis reveals that the intensity of the experience is directly predictive of the degree of anxiogenic-like affective change in behavior. For example, the more cat bites, the more anxious the rat (Adamec, Shallow, & Budgell, 1997). This is consistent with the fact that the nature of the stress experience predicts symptom severity in PTSD patients (Ikin, Sim, Creamer, Forbes, McKenzie, Kelsall, Glass, McFarlane, Abramson, Ittak, Dwyer, Blizzard, Delaney, Horsley, Harrex, Schwarz, 2004; Schnyder, Moergeli, Trentz, Klaghofer, Buddeberg, 2001). Third, dose response-like effects reported in PTSD patients are consistent with animal studies which show reduced impact on rodent ALB by exposure to predator odor as compared to unprotected exposure to a cat (Adamec et al., 1997). Fourth, if one were to use a comparison of ratio of life span, 7.5 days of a rat's 3-year life span would be equivalent to 6 months of a human living 72 years. Therefore, since ALB is increased for at least 3 weeks after predator stress (Adamec & Shallow, 1993), the animal would have experienced chronic anxiety for at least the equivalent of 18 months of a human's life span. This time line meets the criterion as set out by the DSM IV (APA, 1994) in which anxiety is considered chronic if it persists for three months or longer (Adamec, 1997;

Adamec & Shallow, 1993, Adamec et al., 1997). Fifth, this model has neurobiological face validity in that right amygdala and hippocampal circuitry are implicated in behavioral changes produced by predator stress and these brain areas are consistent with those areas thought to be involved in PTSD (Adamec, et al., *in press*). For example, brain imaging studies implicate the anterior temporal lobe (Shin et al., 1997) and in particular, hyperexcitability of the right amygdala in response to script driven trauma reminders in PTSD patients (Rauch, Savage, Alpert, Fischman, & Jenike, 1997; Rauch et al., 1996; Rauch & Shin, 1997; Shin et al., 1997; Shin, McNally, Kosslyn, Thompson, Rauch, Aplert, Metzger, Lasko, Orr, & Pitman, 1999). Sixth, individual differences in vulnerability to the stressor are found in susceptibility to predator stress as well as PTSD. As mentioned above, 50-60% of the population will experience some form of traumatic stress, yet only approximately 15% of people will develop PTSD. Similarly, after predator stress, approximately 25% are high reactors (show heightened ALB), 25% are low reactors (behavior resembles handled control rats), and the remaining 50% fall in between (Cohen, Zohar, Matar, Zeev, Loewenthal, & Richter-Levin, 2004). Seventh, predator stressed rats display an enhanced stress hormonal response when exposed to the elevated plus maze (Adamec, Blundell, Strasser, Burton, accepted). This is consistent with PTSD patients who show elevated cortisol response to non-trauma related stressors. Finally, similar lasting changes in hyper-arousal as measured by startability and habituation of startle are seen in both predator stressed rats and humans with PTSD (Adamec et al., 1998; Adamec, 1997). Furthermore, persistent ALB after predator stress may model the hyper-arousal associated with PTSD (Adamec et al., 2001).

1.6 Behavioral, Physiological, and Molecular Research in Rats after Predator Stress

1.6.1 Behavioral Research

Unprotected exposure to a cat produces a long-lasting increase in rat ALB (Adamec & Shallow, 1993; Adamec et al., 2003; Blundell, Adamec, & Burton, 2005), with some behavioral changes lasting at least three weeks after cat exposure (Adamec, 1997; Adamec & Shallow, 1993) or longer (Cohen et al., 2004). Behavioral effects of predator stress have been evaluated in a number of tests including hole board, EPM, unconditioned response to an acoustic startle, light/dark box, and social interaction. Furthermore, control studies have shown that exposure to cat odor in the cat exposure room is insufficient to induce the robust lasting increases in ALB seen after the unprotected cat exposure (Adamec et al., 1998).

1.6.2 Electrophysiological Research

Electrophysiological studies in rodents suggest that predator stress leads to LTP-like changes in transmission in amygdala afferent and efferent pathways (Adamec et al., 2001; Adamec et al., 2003). In particular, predator stress causes potentiation in neural transmission from the hippocampus via the ventral angular bundle (VAB) to the basolateral amygdala (BLa) and from the Ce to the lPAG (Adamec et al., 2001; Adamec et al., 2003; Adamec, Blundell & Burton, 2005a). As seen in the cat, predator stress-induced LTP-like changes in specific amygdala pathways (Ce-lPAG and VAB-BLa) may mediate specific behavioral changes. For example, path analysis shows that 70-80% of the total variance in behavior in the EPM altered by predator stress is accounted for by

LTP-like changes in amygdala afferent and efferent transmission in the right hemisphere (Adamec et al., 2005a; Adamec et al., 2001). Importantly, as in the cat, stress-induced LTP-like changes in amygdala afferent and efferent pathways are NMDA receptor-dependent (Adamec, Blundell & Burton, 2005b).

1.6.2.1 VAB-BLa Pathway

This pathway provides a monosynaptic input to the BLa from VAB, arising from the entorhinal cortex and ventral subiculum (VAB-BLa) (Maren & Fanselow, 1995). This pathway supports NMDA-dependent LTP, and potentiation in the pathway accompanies contextual fear conditioning (Maren, Aharonov, Stote & Fanselow, 1996).

Predator stress-induced LTP-like changes in the right VAB-BLa pathway have been seen at one, nine, and ten-twelve days post-predator stress (Adamec et al., 2001; Adamec et al., 2003; Adamec et al., 2005). In the left hemisphere however, predator stress-induced long term depression (LTD)-like changes are seen one day post-stress, continuing, although faded, until day nine (Adamec et al., 2001). At ten-twelve days after predator stress, left VAB-BLa transmission is either no different than controls or it is potentiated (Adamec et al., 2005a; Adamec et al., 2005b). One explanation, which will be discussed in further detail below is that in the left VAB-BLa, LTD-like changes compete with and cover LTP-like changes until several days after predator stress (10-12 days) when LTD-like changes decay sufficiently to reveal the LTP-like effect, that may be non-NMDA receptor-dependent. Potentiation in the right VAB-BLa pathway after predator stress, however, is NMDA receptor-dependent.

1.6.2.2 Ce-lPAG Pathway

The PAG, or gray matter that surrounds the aqueduct of Sylvius, is involved in the mediation/modulation of several brain functions, such as nociception, sexual and emotional behavior. In particular, it is implicated in rodent defensive behaviors and in ALB (Brandao, Anseloni, Pandossio, De Araujo, & Castilho, 1999; Davis, 1992; Kemble, Blanchard, & Blanchard, 1990; Kopchia, Altman, & Commissaris, 1992; Makino, Shibasaki, Yamauchi, Nishioka, Mimoto, Wakabayashi, Gold, & Hashimoto, 1999; Moller, Sommer, Thorsell & Heilig, 1999; Rosen & Davis, 1990; Shepard, Barron, & Myers, 2000; Swiergiel, Kalin, Rubin, & Takahashi, 1992). Anatomical and functional data suggest that the PAG can be divided into four longitudinal columns: dorsomedial, dorsolateral, lateral and ventrolateral (Bandler, Carrive, & Depaulis 1991; Bandler & Shipley, 1994; Carrive, 1993).

Single pulse stimulation of the Ce evokes a negative going field potential with a short latency in the lPAG (Adamec et al., 2001; Adamec et al., 2003). The PAG recording site receives monosynaptic projections from the Ce (Rizvi, Ennis, Behbehani, & Shipley, 1991) which have mixed excitatory and inhibitory effects on single PAG cells (Da Costa Gomez, & Behbehani, 1995). Predator stress increases the size of the Ce-lPAG evoked potential one day post-stress in both hemispheres, suggesting a LTP-like change in transmission (Adamec et al., 2001). When Ce-lPAG transmission is examined nine to twelve days after predator stress, LTP-like changes are present in the right, but not the left hemisphere (Adamec et al., 2001; Adamec et al., 2003). This finding is consistent with research in the cat, which shows a changing pattern in amygdalo-lPAG

transmission following stress. As discussed above, potentiation in the right amygdalo-lPAG transmission is longer-lived than potentiation in the left pathway after stress in the cat (Adamec 1997; 1998a; 1999). Furthermore, potentiation in this pathway after predator stress is NMDA receptor-dependent (Adamec et al., 2005b), as is potentiation in the amygdalo-lPAG pathway after FG-7142 in the cat (Adamec, 1998a).

Other research has implicated the right amygdala in fear and anxiety. Coleman-Mesches and McGaugh (1995) have demonstrated that the amygdala in the left and right hemispheres play different roles in the acquisition and expression of fear learning. For example, rats given bilateral infusions of lidocaine in the amygdala prior to the initial training were impaired on acquisition, retention, and subsequently the relearning of the inhibitory learning task later. Unilateral infusions of lidocaine did not affect acquisition, yet rats given lidocaine in the right amygdala demonstrated impaired retention two days later. Furthermore, post-training dopamine infusions into the right BLa, but not the left, enhanced retention on an inhibitory avoidance task (Lalumiere & McGaugh, 2005). Behavioral effects of limbic sensitization produced by kindling also show hemispheric asymmetries. For example, Adamec and colleagues have shown that kindling of the left medial/BLa amygdala is anxiolytic whereas kindling of the right amygdala is anxiogenic (Adamec & Morgan, 1994; Adamec, Blundell, & Burton, 2004). In addition to hemispheric differences, the degree of EPM anxiety following kindling is also dependent on electrode placement in the anterior-posterior (AP) plane (Adamec et al., 2004). For instance, increases in ALB accompany kindling of anterior locations in the right corticomedial amygdala nuclei, whereas more posterior foci are either behaviorally

neutral (medial amygdala) or anxiolytic (cortical nuclei). The reverse appears to occur with kindling of the right central nucleus of the amygdala, with more anterior sites being anxiolytic and more posterior sites being anxiogenic in the EPM.

1.6.3 Physiological and Behavioral Changes After Predator Stress and NMDA Receptors

1.6.3.1 NMDA Receptors

Glutamate is the main excitatory neurotransmitter in the central nervous system (Collingridge & Lester, 1989). Glutamate receptors are broadly classified as ionotropic and metabotropic. Ionotropic glutamate receptor subtypes are further classified according to their specific agonists as AMPA, kainate, and NMDA. Calcium influx triggers enzymatic cascades which lead to lasting changes in synaptic transmission (Massicotte, 2000). Glutamate activates NMDA receptors with a high affinity, and the kinetics of activation and inactivation of the receptor are much slower than for AMPA and kainate (Benveniste & Mayer, 1991; Dingledine, Borges, Bowie, & Traynelis, 1999). It has also been shown that metabotropic glutamate receptors can potentiate NMDA receptor activation (Lan, Skeberdis, Jover, Zheng, Bennett, & Zukin, 2001). The NMDA receptor has many endogenous modulators, including polyamines (spermine and spermidine), histamine, cations (Zn, H, Mg), arachidonic acid and dynorphin (Dingledine et al., 1999). Because the NMDA receptor exhibits a voltage-dependant Mg blockage in its inactivated state, activation requires the partial depolarization of the membrane to remove the Mg ions. Glutamate activation of NMDA receptors also requires the binding of glycine (GLY) to the strychnine-insensitive GLYb site; thus, GLY acts as an essential

co-agonist (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). These unique features make the NMDA receptor a target for the action of many drugs including agonists, competitive antagonists, uncompetitive blockers (that block the ion channel in the open-state) and non-competitive antagonists (that block the ion channel in the resting state). The NMDA receptor complex is involved in many functional processes including learning and memory (Morris, 1989), neural development and synaptic plasticity (Cotman, Monaghan, & Ganong, 1988), neural injury after ischemia or hypoglycemia (Oguro, Miyawaki, Cho, Yokota, Masuzawa, Tsubokawa, & Kawai, 1997), epilepsy and other chronic neurodegenerative disorders (Meldrun, 1985), drug dependence and tolerance (Marek, Ben-Eliyahu, Vaccarino, & Liebeskind, 1991), neuropathic pain (Eisenberg & Pud, 1998), and anxiety and depression (Matheus, Nogueira, Carobrez, Graeff, & Guimaraes, 1994; Adamec et al., 1999a).

1.6.3.2 Electrophysiological Research

The enhanced potentiation of amygdala afferent and efferent transmission after predator stress is blocked by an NMDA receptor antagonist, CPP (3-(2-carboxypiperazin4-yl)propyl-l-phosphonic acid) (Adamec, Blundell, & Burton, 2005b), consistent with research in the cat (Adamec, 1998). Specifically, NMDA receptor block prevents predator stress-induced potentiation of the right VAB-BLa and right Ce-lPAG evoked potentials (Adamec et al., 2005b). Interestingly, in the left VAB-BLa, an NMDA receptor antagonist increases potentiation (Adamec et al., 2005b). As mentioned above, LTD-like changes were seen in the left VAB-BLa nine days after predator stress and this

was replaced by a LTP-like change ten days post-stress (or no difference between predator stressed and controls) (Adamec et al., 2003; Adamec et al., 2005a). The authors suggest that there is an NMDA receptor-dependent form of LTD fades, revealing a non-NMDA receptor-dependent LTP-like change. Thus, the NMDA receptor antagonist blocks the LTD-like change and allows full expression of the LTP-like change. Of course, further studies are necessary to test this hypothesis.

1.6.3.3 Behavioral Research

Like stress-induced LTP, most changes in ALB following predator stress are also NMDA receptor-dependent. Systemic administration of both competitive and non-competitive NMDA receptor antagonists before, but not after, predator stress prevent lasting changes in ALB (Adamec et al., 1999a; Adamec et al., 1999b; Blundell et al., 2005 - Chapter 2; Cohen, Zohar, Matar, 2003). Moreover, a local NMDA receptor antagonist in the amygdala blocks some, but not all, of the behavioral changes produced by predator stress in rats (Adamec et al., 1999a). For instance, local injection of the NMDA antagonist MK-801, in the right dorsolateral amygdala, 30 minutes prior to cat exposure prevented the expected increase in acoustic startle measured one week later. However, right amygdala injections of MK-801 did not prevent decreased open arm exploration and decreased risk assessment in the EPM. In contrast, MK-801 injections into the left dorsolateral amygdala 30 minutes prior to cat exposure prevented the expected decreases in risk assessment in the EPM measured one week later, while leaving intact decreases in open arm exploration and increases in acoustic startle response. Taken

together, these findings are consistent with the view that NMDA receptor-dependent LTP-like changes in particular amygdala circuits may underlie particular behavioral changes in response to stress.

In addition to the amygdala, NMDA receptors in the PAG appear to be involved in anxiety. The main excitatory input to the PAG is glutamatergic and NMDA receptors are distributed throughout the PAG (Albin, Makowiec, Hollingsworth, Dure, Penney, & Young, 1990). Bandler (1982) first reported defensive "rage" following microinjection of glutamate within the dorsal PAG (dPAG) of freely moving cats and mydriasis, retraction of the ear, vocalization and hissing in head restrained cats. Furthermore, in cats, the microinjection of the non-selective NMDA/GLYb receptor antagonist, kynurenic acid or AP7, into the dPAG, blocked the defensive reaction induced by electrical stimulation of the dorsal medial hypothalamus (DMH) (Lu, Shaikh, & Siegel, 1992; Schubert, Shaikh, & Siegel, 1996). Since then, a growing body of evidence has extended these observations to other species. For example, AP7 injected into the dPAG is anxiolytic in rats, as detected by increased open arm activity in the EPM (Guimaraes, Carobrez, de Aguiar, & Graeff, 1991; Molchanov & Guimaraes, 2002). In addition, the rodent PAG is activated by predator stress. Specifically, relative to controls, rats exposed to a cat exhibit increased Fos-like immunoreactivity (lir) in specific medial hypothalamic and PAG sites (Canteras, Chiavegatto, Valle, & Swanson, 1997; Canteras and Goto, 1999). In addition, Dielenberg, Hunt, and McGregor (2001b) found that cat odor-exposed rats showed robust Fos-lir in the dorsomedial, dorsolateral and ventrolateral PAG. Furthermore, microinjection of the benzodiazepine agonist, midazolam, into the

dorsal PAG causes anxiolytic-like effects (Russo, Guimaraes, de Aguiar, & Graeff, 1993).

1.6.4 Molecular Changes After Predator Stress

1.6.4.1 Cyclic AMP Response Element Binding Protein

The 43-kDa cAMP-response-element-binding protein (CREB) is a member of a family of proteins that function as transcription factors. Localized within the nucleus, transcription factors such as CREB are crucial for stimulus-transcription coupling: the transmission of events that occur at cell membranes into alterations in gene expression. In turn, altering gene expression by regulating the expression of virtually all types of neural proteins, can ultimately affect the function of individual neurons and entire neuronal circuits. Numerous intracellular signaling pathways are involved in transmitting information initiated by membrane receptor-mediated actions to the cell nucleus, where they interact with CREB to trigger processes that culminate in gene transcription. The key steps involved in CREB-mediated gene transcription include phosphorylation, dimerization, and binding at response elements in DNA (Mayr & Montminy, 2001; Shaywitz & Greenberg, 1999).

CREB is common to the signaling cascades that are initiated following stimulation of several neurotransmitter receptors, and is regulated by phosphorylation by cAMP-dependent protein kinase A (PKA) and by Ca2+ -calmodulin-dependent protein kinases (CaMKs) (Lonze & Ginty, 2002; Sheng, Thompson, & Greenberg, 1991). CREB is also phosphorylated by ribosomal S_6 kinase (RSK) via mitogen-activated protein kinase

(MAPK) (Arthur, Fong, Dwyer, Davare, Reese, Obrietan, & Impey, 2004). pCREB activity can be blocked by repressors such as the inducible cAMP early repressor (Molina, Foulkes, Lalli, & Sassone-Corsi, 1993), or by dephosphorylation catalyzed mainly by the nuclear protein phosphatase 1 (Hagiwara, Alberts, Brindle, Meinkoth, Feramisco, Deng, Karin, Shenolikar, & Montminy, 1992). pCREB bound to CRE sites in the promotor regions of early response genes (Sheng & Greenberg, 1990) acts in combination with other CREB family transcription factors to recruit the adaptor molecular CBP (CREB binding protein, Arias, Alberts, Brindle, Claret, Smeal, Karin, Feramisco, & Montminy, 1994; Kwok, Lundblad, Chrivia, Richards, Bachinger, Brennan, Roberts, Green, & Goodman, 1994). CBP then interacts directly with the transcriptional apparatus, inducing gene expression. Target genes are immediate early genes as well as later response genes, which are induced over periods of hours to days and encode for growth factors, enzymes, and neurotransmitters (Robertson, 1992).

1.6.4.2 Roles of CREB in Complex Behaviors

Substantial evidence suggests that CREB/pCREB plays an important role in many complex behaviors including the formation and consolidation of long-term memories (Bailey, Bartsch, & Kandel, 1996; Frank & Greenberg, 1994; Josselyn, Shi, Carlezon, Neve, Nestler, Davis, & 2001; Kida, Josselyn, de Ortiz, Kogan, Chevere, Masushige, & Silva, 2002; Silva, Kogan, Frankland, & Kida, 1998; Stevens, 1994), drug addiction (Carlezon, Thome, Olson, Lane-Ladd, Brodkin, Hiroi, Duman, Neve, Nestler, 1998; Guitart, Thompson, Mirante, Greenberg, & Nestler, 1992; Maldonado, Blendy, Tzavara,

Gass, Roques, Hanoune, & Schutz, 1996; McClung & Nestler, 2003; Nestler, 2004; Olson, Zabetian, Bolanos, Edwards, Barrot, Eisch, Hughes, Self, Neve, & Nestler, 2005; Walters & Blendy, 2001), and emotional disorders (Adamec et al., 2003; Adamec et al., in press; Chen, Shirayama, Shin, Neve, & Duman, 2001; Conti, Cryan, Dalvi, Lucki, & Blendy, 2002; Duman, Heninger, & Nestler, 1997; Jeon, Seong, Juhnn, Kang, Ha, Kim, & Park, 1997; Newton, Thome, Wallace, Shirayama, Schlesinger, Sakai, Chen, Neve, Nestler, & Duman, 2002; Nibuya, Nestler, & Duman, 1996; Pandey, Roy, & Zhang, 2003; Thome, Sakai, Shin, Steffen, Zhang, Impey, Storm, & Duman, 2000; Wallace, Stellitano, Neve, & Duman, 2004).

1.6.4.2.1 CREB and Long Term Memory

CREB function in long term memory has been shown in a variety of animals including *Aplysia*, *Drosophila*, mice and rats. For example, serotonin, which causes long-term facilitation of the gill-withdrawal reflex in *Aplysia*, was shown to activate transcription of a reporter gene artificially driven by CREs in a CREB-dependent fashion (Kaang, Kandel, & Grant, 1993). In *Drosophila*, expression of CREB activator isoform facilitates long-term memory (Yin, Wallach, Del Vecchio, Wilder, Zhou, Quinn, & Tully, 1994). Mutant mice lacking CREB isoforms α and δ have deficiencies in long-term retention of several learning tasks including contextual fear conditioning, Morris water maze and socially transmitted food preference, and markedly attenuated LTP (Bourtchuladze, Frenguelli, Blendy, Cioffi, Schutz, & Silva, 1994; Kogan, Frankland, Blendy, Coblentz, Marowitz, Schutz, & Silva, 1997). In rats, interruption of CREB

signaling with antisense oligodeoxynucleotides directed against CREB mRNA in the hippocampus results in impairment of memory formation for water maze training (Guzowski & McGaugh, 1997). In addition, infusions of antisense oligodeoxynucleotides to CREB directly into the rat amygdala impair long-term conditioned taste aversion memory (Lamprecht, Hazvi, & Dudai, 1997). Moreover, overexpression of CREB using viral-mediated gene transfer within the amygdala of rats exposed to social defeat enhanced subsequent defeat-induced changes in social behavior (Jasnow, Shi, Israel, Davis, & Huhman, 2005). Finally, fear-associated learning increases pCREB levels and CRE-mediated transcription in the hippocampus (Impey, Smith, Obrietan, Donahue, Wade & Storm, 1998; Taubenfeld, Wiig, Bear, & Alberini, 1999).

1.6.4.2.2 CREB and Addiction

CREB-mediated gene expression in three main brain areas, including the locus coeruleus (LC), nucleus accumbens (NAc), and ventral tegmental area (VTA), has been implicated in addiction. For example, morphine inhibits CREB phosphorylation in the LC (Guitart et al., 1992), a brain area important for opiate physical dependence and withdrawal (Aghajanian, 1978; Koob, Maldonado, & Stinus, 1992). In addition, blockade of CREB function within the LC reduces expression of CREB-regulated target genes, and reduces the electrophysiological and behavioral markers of opiate physical dependence and withdrawal (Lane-Ladd, Pineda, Boundy, Pfeuffer, Krupinski, Aghajanian, & Nestler, 1997). Furthermore, $CREB^{\alpha\delta-}$ deficient mice show attenuated opiate physical withdrawal (Maldonado et al., 1996).

Alterations in CREB expression in the NAc have also been associated with drug addiction. Direct activation of PKA activity, which increases phosphorylation of CREB, within the NAc reduces the rewarding effects of cocaine, whereas PKA inhibition has the opposite effect (Self, Genova, Hope, Barnhart, Spencer, & Nestler, 1998). In addition, increasing CREB reduces the rewarding effects of cocaine (Carlezon et al., 1998).

Finally, alterations in CREB function in the VTA, another brain area associated with the rewarding effects of opiates (Bozarth & Wise, 1981), also affect addictive behaviors (Olson et al., 2005). Importantly, the consequences of alterations in CREB function in this area appear to be dependent on the anatomical localization of gene transfer. Specifically, increased CREB in rostral portions of the VTA increases the rewarding effects of cocaine and morphine, whereas similar changes in the caudal portion have the opposite effects.

1.6.4.2.3 CREB and Emotional Disorders

In addition to memory and drug addiction, CREB/pCREB function has been associated with depression and anxiety. Elevations in CREB can either increase or decrease depressive-like symptoms, depending on the brain area. For example, increasing CREB using viral-mediated gene transfer in the hippocampus has antidepressant-like effects in rodents (Chen et al., 2001). In the amygdala, however, alterations in CREB function in models of depression appear to be state-dependentFor instance, increasing CREB (using the viral vector-mediated gene transfer approach to increase the expression of CREB) in the amygdala before training in the learned

helplessness paradigm causes prodepressive-like effects, whereas expression after training results in antidepressive-like effects (Wallace et al., 2004). Furthermore, increased numbers of pCREB stained cells were found in several amygdala nuclei in patients with mood disorders who committed suicide (Young, Bezchlibnyk, Chen, Wang, & MacQueen, 2004).

There is also evidence that CREB/pCREB play a role in ALB. For example, Wallace et al. (2004) found that rats made to over-express CREB in the BLa were more anxious in the EPM. In addition, changes in ALB have been assessed after EPM using a PKA inhibitor (to reduce pCREB) infused into the Ce or BLa one-hour prior to exposure to the EPM. Rats given a PKA-inhibitor into the Ce, but not the BLa, showed increased ALB in the EPM (Pandey, Roy, & Zhang, 2003). This study does not appear to be consistent with that of Wallace et al. (2004) in which increased ALB was produced when CREB was over-expressed (via viral vector-mediated gene transfer approach to increase CREB) in the BLa. However, as suggested by Wallace et al. (2004), this discrepancy may be a result of acute (PKA) versus chronic (viral expression) alteration of CREB function. In addition, Hebda-Bauer, Watson, & Akil, (2004) found that $CREB^{\alpha\delta-}$ deficient mice show no changes in ALB as measured in the EPM.

1.6.4.3 pCREB After Predator Stress

Changes in pCREB-lir have also been assessed after predator stress. Exposure to a cat induces changes in pCREB-lir in the PAG and amygdala, but not in the ventral medial hypothalamus. Specifically, predator stress increased pCREB-lir in right lateral

column of the PAG (Adamec et al., 2003) and several amygdala nuclei including the basomedial (BM), BLa, Ce, and La (Adamec et al., *in press*). Increases in pCREB-lir have also been assessed in the amygdala after fear potentiated startle (Meloni, Jackson, Cohen & Carlezon, 2003) and electric shock (Stanciu, Radulovic & Spiess, 2001).

In addition to changes in pCREB, c-*fos* has been examined as a marker of neural activity in brain areas after exposure to predator stress. c-*fos* is an immediate early gene product that is inducible via CRE-mediated transcriptional activation (Herdegen & Leah, 1998; Herrera & Robertson, 1996; Morgan & Curran, 1991). Consistent with the pCREB data, exposure to a cat or predator odors increases c-*fos*-lir in the PAG and amygdala of rats (Canteras & Goto, 1999; Day, Masini, & Campeau, 2004; Dielenberg et al., 2001a; Dielenberg et al., 2001b, McGregor, Hargreaves, Apfelbach, & Hunt, 2004). In contrast to pCREB data, predator stress (odor) activation of c-*fos* is restricted to the medial amygdala and does not appear to be increased in lateral or basal nuclei of the amygdala. Since predator odor does not produce long-lasting anxiogenic effects in the EPM (unlike unprotected exposure to a cat), Adamec et al. (2005a) have suggested that lasting changes in ALB following predator stress may require activation and lasting sensitization of neural circuitry involved in the storage of lasting fear memories. This sensitization may not be produced by predator odor alone.

1.7 Justification for Studies

1.7.1 Chapter 2 - Role of NMDA Receptors in the Syndrome of Behavioral Changes Produced by Predator Stress

As discussed above, predator stress-induced changes in ALB are NMDA receptor-dependent (Adamec et al., 1999a, 1999b). Thus, the purpose of the first experiment, described in Chapter 2, was to extend the initial findings that the competitive NMDA receptor antagonist, CPP, blocks the effects of predator stress on ALB. This study examined a wider range of doses in a dose response analysis of CPP on ALB in predator stressed rats. In addition, we tested CPP on an increased number of behavioral tests because there is growing evidence for separable neural substrates mediating the effects of stress on affect (Adamec, 1997; Adamec et al., 2001; Adamec et al., 1999a; Adamec et al., 2003).

1.7.2 Chapter 3 - The NMDA Receptor Antagonist CPP Blocks the Effects of Predator Stress on pCREB in Brain Regions Involved in Fearful and Anxious Behavior

NMDA receptor antagonists given prior to predator stress block both increases in ALB and potentiation of amygdala neural transmission (Adamec et al., 2005b; Adamec et al., 1999a). Since phosphorylation of CREB is regulated by NMDA receptors (Segal & Murphy, 1998) and pCREB expression is increased after predator stress (Adamec et al., 2001; Adamec et al., 2003; Adamec et al., *in press*), the purpose of the second study (detailed in Chapter 3) was to determine whether an NMDA receptor antagonist (CPP) that blocks behavioral and electrophysiological effects of predator stress, can also block

the predator stress-induced enhancement of pCREB-lir. We assessed pCREB-lir after predator stress in a larger number of brain areas including the amygdala, PAG, BNST, DMH, and ACC.

1.7.3 Chapter 4 - Elevated pCREB in the PAG After Exposure to the Elevated Plus Maze in Rats Previously Exposed to a Cat.

As discussed above, predator stress induces increases in pCREB-lir in various brain areas. One of the goals of this study is to determine whether a different, milder stressor will elicit a similar pattern of neural plastic change. Are the changes in pCREB-lir a general response to any stressor, or is it specific to predator stress? To address this question, we examined pCREB lir after exposure to the EPM, a mild stressor, in brain areas that showed pCREB changes after predator stress (PAG, amygdala and BNST).

The second goal of this study was to determine whether there is an enhanced stress sensitization in the neuroplastic response to the EPM in animals previously exposed to a cat. To address this issue, pCREB-lir was examined in the amygdala, the PAG, and the BNST after exposure to the elevated plus maze in naïve animals and in animals exposed to a cat.

Co-Authorship Statement

As this thesis is submitted in Manuscript (Publication) Format, a statement is necessary to address the specific contributions of intellectual and practical contribution made by the primary author.

The origins of the research proposals were conducted jointly between my Ph.D. supervisor, Dr. Robert Adamec, and me. All other work found within this text was conducted by myself (with one exception - see below). My contributions include inception of the experimental design, data collection and analysis, and preparation of the manuscripts for publication.

In the second chapter (Role of NMDA Receptors in the Syndrome of Behavioral Changes Produced by Predator Stress), Paul Burton, Dr. Adamec's research assistant, helped with some of the behavioral testing.

CHAPTER 2:

Role of NMDA Receptors in the Syndrome of Behavioral Changes Produced by Predator Stress

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2.1 Abstract

The effects of CPP (3-(2-carboxypiperazin4-yl)propyl-l-phosphonic acid), an *N*-methyl-*D*-aspartate (NMDA) receptor antagonist, were examined in predator stressed rats. An affect test battery assessed the behavioral response to stress and employed hole board, elevated plus maze (EPM), light/dark box, social interaction, social avoidance and response to acoustic startle tests. Doses of 1-10 mg/kg of CPP administered ip 30 min prior to predator stress blocked the effects of predator stress on some, but not all behaviors, measured eight-nine days later. A dose of 10 mg/kg of CPP was required to block most behaviors except habituation to startle, which was blocked with 1 or 10 mg/kg of CPP. Behaviors in which effects of predator stress were not blocked by CPP included reduction in unprotected head dips in the EPM and reduced social interaction. In addition, predator stress did not affect social avoidance behavior, as measured with the Haller test. These findings extend previous work showing NMDA receptor dependence of effects of predator stress on behavior in the EPM and on amplitude of acoustic startle response. Novel findings include NMDA receptor dependence of predator stress effects on light/dark box behavior and startle habituation. Taken together, the findings add to a body of evidence showing that a syndrome of behavioral changes follows predator stress. Components of this syndrome of behavioral changes likely depend on changes in separable neural substrates initiated by NMDA receptors as well as by other neurochemical means.

2.2 Introduction

Anxiety associated with traumatic stress is a serious problem in view of the fact that 61% males and 51% of females in North America experience some form of traumatic stress in their lifetimes (Kessler, Sonnega, Bromet, Hughes & Nelson, 1995) and of those, 15% may develop posttraumatic stress disorder (PTSD) (Kessler, McGonagle, Zhao, Nelson, Hughes, Eshleman, Wittchen, Kendler, 1994). PTSD can be a debilitating disorder characterized by severe anxiety, nightmares, agitation and often depression. Because there is no cure for this disorder, the study of the effects of stress on brain function is important.

While there is no ideal animal model to study the mechanisms of stress precipitation of affective disorder, there are several that are promising. Studies of classical conditioning of freezing have advanced our understanding of mechanisms of neuroplasticity underlying acquisition and extinction of enhanced fear responses to simple and complex sensory stimuli (contextual conditioning; Blair, Schafe, Bauer, Rodrigues, & LeDoux, 2001; Maren, De Oca, & Fanselow, 1994; Rogan, Stäubli, & LeDoux, 1997; Schafe, Nader, Blair, & LeDoux, 2001). In addition, there is growing interest in using this model to gain insights into mechanisms of onset of PTSD as a guide to post-stressor prophylactic intervention in humans (Pitman, Sanders, Zusman, Healy, Cheema, Lasko, Cahill, & Orr, 2002). Furthermore, this model has been used to understand and suggest improvements for the therapeutic outcome of exposure therapies (Myers & Davis, 2002).

The classical conditioning model confirms the importance of neuroplasticity and long term potentiation (LTP) in amygdala circuitry in both conditioning and extinction of fear (Bauer, LeDoux, & Nader, 2001; Blair et al., 2001; Myers & Davis, 2002; Nader, Schafe, & LeDoux, 2000; Schafe, Atkins, Swank, Bauer, Sweatt, & LeDoux, 2000; Schafe & LeDoux, 2000; Schafe et al., 2001). Moreover, these forms of learning are NMDA receptor-dependent (Baker & Azorlosa, 1996; Campeau, Miserendino, & Davis, 1992; Davis, 2002; Decola, Kim, & Fanselow, 1991; Fanselow, Kim, & Yipp, 1992; Kim, Decola, Landeira-Fernandez, & Fanselow, 1991; Lee, Choi, Brown, & Kim, 2001; Lee & Kim, 1998; Stiedl, Birkenfeld, Palve, & Spiess, 2000), as are some, but not all, forms of amygdala neuroplasticity associated with acquisition and extinction of fear conditioning (Bauer, Schafe, & LeDoux, 2002; Davis, 2002; Goosens & Maren, 2002; Lee, Lee, & Choi, 2002; Royer & Pare, 2002; Tsvetkov, Carlezon, Benes, Kandel, & Bolshakov, 2002). While substantial progress has been made in uncovering the neural substrates of conditioned fear, the link of conditioned fear models to stressor-induced psychopathology is indirect. The work of Roozendaal and McGaugh provides a more direct link by showing how stress-induced neuroendocrine response impacts amygdala circuitry involved in associative fear conditioning (McGaugh & Roozendaal, 2002; Roozendaal, 2002). Specifically, release of norepinephrine and activation of β-adrenoceptors within the basolateral amygdala is critical in mediating adrenal stress hormone regulation of memory consolidation. Their findings may explain how stressors produce the indelible fear memories associated with PTSD.

It is important to note, however, that not all affective disorders associated with severe stress are explicable by associative fear conditioning. Sensitized fearfulness manifested as generalized anxiety is also a feature of PTSD (Pitman, 1997). Therefore, animal models of such changes are also relevant to stress precipitation of affective disorder. Moreover, animal models with ecological validity with regard to stressors are important to both validate and extend the findings of conditioning models.

Studies of lasting changes in affect following exposure to species-relevant life threatening circumstances provide models of stressor-induced affective psychopathology with ecological validity. Exposure of rodents to predator stimuli (predator stress) is fear provoking and stressful (Adamec, 1999; Blanchard, Nikulina, Sakai, McKittrick, McEwen, & Blanchard, 1998; Dielenberg, Carrive, & McGregor, 2001a; McGregor, Schrama, Ambermoon, & Dielenberg, 2002). Moreover, predator stress (exposure to a cat) lastingly increases rodent anxiety-like behavior (ALB) when the exposure is unprotected and inescapable (Adamec, 1997; Adamec & Shallow, 1993). Behavioral changes are detectable in several tests of anxiety including elevated plus maze (EPM), light/dark box, social interaction and response to acoustic startle (Adamec, 2003). Some effects on behavior last at least three weeks after predator stress (Adamec & Shallow, 1993). In addition, multivariate correlation analysis (path analysis) reveals that both the nature of the stressor (cat behavior toward the rat) and the defensive response of the rat to the cat are predictive of the degree of anxiogenic response measured one week later (Adamec, Kent, Anisman, Shallow, & Merali, 1998). Nature of response to traumatic stressors, as well as the severity of the stressor, is also predictive of symptom severity in

PTSD (Ikin, Sim, Creamer, Forbes, McKenzie, Kelsall, Glass, McFarlane, Abramson, Ittak, Dwyer, Blizzard, Delaney, Horsley, Harrex, & Schwarz, 2004; Marmar, Weiss, Schlenger, Fairbank, Jordan, Kulka, & Hough, 1994; McNally, 2003).

Parallels exist between mechanisms of fear conditioning and neural mechanisms underlying behavioral effects of unprotected predator stress. For example, lasting changes in behavior induced by predator stress are NMDA receptor-dependent (Adamec, Burton, Shallow, & Budgell, 1999a; Adamec, Burton, Shallow, & Budgell, 1999b). Specifically, systemic administration of competitive and non-competitive NMDA receptor blockers 30 min before, but not 30 min after, predator stress prevents lasting changes in ALB measured in the EPM. Moreover, local NMDA receptor block in the amygdala 30 min prior to predator stress prevents increases in ALB as measured in the EPM and response to acoustic startle. However, which behavioral change is prevented depends on the hemisphere of the injection (Adamec et al., 1999b). The observation that injection prior to stress, but not after, prevents behavioral change is consistent with the hypothesis that NMDA receptor-dependent LTP-like changes underlie the behavioral changes.

The purpose of this study was to extend initial findings that the competitive NMDA receptor antagonist, CPP (3-(2-carboxypiperazin4-yl)propyl-l-phosphonic acid), blocks the effects of predator stress on ALB. CPP is a competitive NMDA receptor blocker that interferes with experimentally- and experientially-induced LTP and neuroplasticity (Abraham & Mason, 1988; Ekstrom, Meltzer, McNaughton, & Barnes, 2001; Hernandez, Derrick, Rodriguez, & Martinez, 1994). Previous studies in this lab showed that a 10 mg/kg dose of CPP blocks behavioral effects of predator stress

(Adamec et al., 1999a). This dose is well above the ip ED50 for blocking central NMDA receptor dependent processes (Lehmann, Schneider, McPherson, Murphy, Bernard, Tsai, Bennett, Pastor, Steel, Boehm, et al., 1987). The present study examined a wider range of doses in a dose response analysis of the effects of CPP on the impact of predator stress on rodent ALB. Previous studies of CPP examined the EPM behaviors of open arm avoidance and risk assessment. Since there is growing evidence for separable neural substrates mediating the effects of stress on affect (Adamec, 1997; Adamec, Blundell, & Collins, 2001; Adamec, 1999; Adamec, Blundell, Burton, 2003), it is also important to test CPP against stress effects on a wider variety of behavioral measures of rodent ALB. In addition to the recently expanded battery of tests (Adamec et al., 2001; Adamec et al., 2003), a novel social avoidance test was added. Social avoidance is a common symptom of anxiety disorder (Johnson, LaVoie, Spenceri, & Mahoney-Wernli, 2001), and is increased by anxiogenic drugs in animals (Cutler, 1993). Moreover, Haller and colleagues (Haller & Bakos, 2002; Haller, Leveleki, Baranyi, Mikics, & Bakos, 2003) have shown that both social defeat and electric shock induce long-lasting social avoidance in rats. The present study assessed the effects on social avoidance of predator stress and the involvement of NMDA receptors in those effects.

2.3 Methods

2.3.1 Subjects and Groups

One hundred thirty male hooded Long Evans rats (Rattus norvegicus) from The Charles River Breeding Farms, Quebec, were used. All rats were housed alone in clear

polycarbonate cages measuring 46 cm x 24 cm x 20 cm for at least four days before testing began. Rats were given food and water *ad lib* and they were exposed to a 12-hour light/dark cycle with lights on at 7 AM. The rats weighed approximately 120 g on arrival and 166 + 0.8 g (mean + SEM) at the beginning of testing. All rats were handled in the same room as their home cages for one minute a day for three days prior to testing. Handling involved picking the rat up with a gloved hand and gently holding it on the forearm. A minimal amount of pressure was used if the rat attempted to escape and the grip was released as soon as the animal became still.

One hundred twenty animals were randomly assigned to one of six groups (N=20 per group). Except for the handled control, all groups were exposed to a cat for five minutes as described elsewhere (Adamec et al., 2001; Adamec & Shallow, 1993). Groups were: handled control, predator stressed only (exposed to a cat), predator stressed plus vehicle or predator stressed plus .1, 1.0, or 10 mg/kg of CPP. Two rats per group were tested each week. Ten rats were used only as stimulus rats for the Haller social avoidance test (described below).

CPP doses were dissolved in 0.5 ml of vehicle (saline). Rats in the vehicle control group were injected with 0.5 ml of saline. All injections were given ip 30 min prior to predator stress.

2.3.2 Predator Stress Testing

Cat exposures occurred between 9 AM and 11 AM. Rats in the predator stressed groups were weighed and then experienced one unprotected exposure to one of four adult

male cats. Cat used and time of test were counterbalanced in all groups to ensure similar exposure of rats in the different groups. Rats were exposed to a cat in a large enclosed room with carpet on the floor. The five minute exposure was videotaped to capture the activities of both the cat and the rat. Cat response to the rat ranged from watching the rat at a distance, to approach and sniffing, with the occasional mild attack. Sometimes the cat pawed and bit a rat but did not physically injure it. All rats were examined for wounds after the test, and none were observed. On the day of predator stress testing, rats in the handled control group were weighed and then handled for one minute. After treatments, all rats were returned to their home cages and left undisturbed until behavioral testing.

2.3.3 Behavioral Measures Taken From Cat Exposures

Behavior of the rat and cat was videotaped and later analyzed to provide a measure of the cat exposure experience among the groups. Cat behaviors scored were; frequencies of approaches to the rat, latency to approach and time spent near the rat, latency to sniff and time spent sniffing the rat, latency to bite and frequency of bites and pawing of the rat. The floor of the exposure room was divided into one foot squares with masking tape. Time spent near the rat was scored when the cat was within one foot of the rat.

The responses of the rat were also recorded and analyzed. Frequencies of active, passive and escape defensive responses were measured. Active defense was scored in several ways: rat initiated approaches to the cat (active approach); rat bites; upright

postures with or without pushing the cat with a forepaw; and rat vocalizations. Passive defense was scored when the rat became immobile for 1 sec or more when the cat approached or remained near. Escape was scored whenever there was a rapid movement of the rat away from the cat when the cat approached or was near.

2.3.4 Post-Treatment Behavioral Testing and Behavioral Measures

Eight and nine days after treatment, behavioral effects of predator stress and handling were assessed. ALB, activity and exploration were measured with the hole board and EPM tests. The light/dark box test was used because it has been employed in mice and rats as a test of ALB (Bilkei, Gyertyán, & Lévay, 1998; Griebel, Belzung, Perrault, Sanger, 2000). Social anxiety was assessed using two tests: the social interaction test (File, 1980), which has been used in previous studies of predator stress (Adamec et al., 2003); and the Haller social avoidance test (Haller & Bakos, 2002). Response to acoustic startle in a lighted startle chamber was also measured. All post-predator stress behavior testing took place in rooms different from the cat exposure room. Importantly, a cat had never been in these testing rooms. The same testing room was used for all groups for a given test.

Social interaction, Haller social avoidance and acoustic startle testing were done on day eight after predator stress while light/dark box, hole board and EPM testing were done on day nine.

The order of testing on day eight was as follows. Social interaction was tested first followed in 5 minutes by the Haller social avoidance test. Approximately two hours

later rats were startle tested for 30 min. Startle testing was done last to avoid any carry over effects to other tests on that day. On day nine, the first test was the light/dark box followed an hour later by the hole board and EPM tests. Testing occurred between 8 AM and 2 PM and time of testing was counterbalanced among groups. Test procedures and associated measures are detailed below.

2.3.4.1 Hole Board and Elevated Plus-Maze

The hole board was used in conjunction with the EPM to provide independent measures of activity and exploratory tendency (File & Wardill, 1975a; File & Wardill, 1975b). The EPM was used to assess ALB. Behavior was videotaped remotely and later analyzed from tape. Hole board and EPM apparatuses are described in Appendix 1. Rats were first placed in the center of the hole board. At the end of the 5 minute hole board test, rats were transferred by gloved hand to the EPM for a further 5 minutes of testing. All rats were placed facing the same open arm of the maze. After the test, rats were returned to their home cages. Both the hole board and EPM were cleaned after each test with a 70% alcohol solution and wiped dry.

Two classes of behaviors were measured in the hole board: activity and exploratory behavior. Activity was scored as frequency of rearing and time spent in motion of any kind (time active). Exploratory tendency was also measured as the number of head dips (placing the snout or head into a hole in the floor) (File & Wardill, 1975b). Time spent near the wall, a measure of thigmotaxis and ALB in some studies, and time spent in the center of the hole board were also recorded as described elsewhere (Adamec,

2001; Adamec & Shallow, 1993). Number of fecal boli deposited in the hole board was counted after each test.

A variety of behavioral measures in the EPM were analyzed from videotape. Two measures that assess open arm exploration in the EPM were recorded: ratio time and ratio entry. Ratio time was the time spent in the open arms of the maze divided by the total time spent in any arm of the maze. The smaller the ratio, the less open arm exploration and the more "anxious" the rat. Ratio entry was the number of entries into the open arms of the maze divided by the total entries into any arm of the maze. The smaller this ratio, the more "anxious" the rat.

In addition to the more standard measures of open arm exploration, we included extra measures derived from the ethological analysis of rodent behavior in the EPM of Rodgers and Johnson (1995). Three kinds of head dips were defined; protected, center, and unprotected. These were scored when a rat dipped its head over the side of an open arm with its hindquarters in a closed arm of the maze (protected), or when a rat was standing with all four feet in the center of the maze (neither in the open or closed arms, center), and when the rat had all four feet in the open arms (unprotected). Protected, center, and unprotected frequencies of rearing and time spent grooming were also measured. In addition, numbers of closed arm entries were scored as measures of exploration/activity in the EPM (Adamec, 2001).

We also examined risk assessment. Risk assessment was scored when a rat poked its head and possibly its forepaws into an open arm of the maze while its hindquarters were in a closed arm of the maze. This behavior resembles the protected stretch attend

posture of Rodgers and Johnson (1995). Frequency of risk assessment was scored. Frequencies were divided by time spent in the closed arm of the maze to produce a relative frequency risk assessment measure. Finally, number of faecal boli deposited in the EPM was counted.

2.3.4.2 Light/Dark Box

The light/dark box was constructed as described in Appendix 1. Briefly, it a single alley apparatus with two chambers, a lighted white chamber and a darkened black chamber. Rats were placed in the apparatus in the white chamber facing away from the dark chamber and allowed to explore both white and black chambers freely for 5 minutes. Behavior was videotaped with a camera mounted above the light/dark box. Two light/dark boxes were constructed allowing testing of two rats at the same time. Boxes used for testing experimental and control groups were counterbalanced. After each test, the box was cleaned with a 70% alcohol solution and wiped dry.

Behaviors measured from videotape included times spent in light and dark chambers, as well as number of entries into either chamber. A rat was considered to be in a chamber when all four feet were within its boundaries. Latency to first leave (escape) from the lighted chamber was also recorded. Finally, number of faecal boli deposited in either chamber was counted.

2.3.4.3 Social Interaction

The test took place in a novel apparatus of the same dimensions as the hole board test apparatus (see Appendix 1), only it had a solid wooden floor and was painted a different color (grey). At the beginning of the test, rats were placed in pairs in the center of the apparatus and videotaped from above for 5 min. One rat was marked on its two sides with a non-toxic black marker to distinguish it from the other rat. Pairs of rats were weight matched as far as possible and pairing was by random assignment. Rats were tested under red light.

Measures taken from videotape for each rat in a pair included time spent interacting with the partner rat (time social interaction), time immobile (freezing for 1 sec or more) when near and when apart from the partner rat, latency to initiate social interaction, number of fights (involving wrestling, upright boxing postures, biting), number of defensive behaviors (sideways defensive postures, laying on back), number of withdrawals (fleeing from the partner during interaction), and number of pursuits (following a partner immediately after it withdraws). A five minute test was used here because recent studies have shown that differences in social interaction produced by predator stress are detectable in the first five minutes of a 10 minute test (Adamec et al., 2003).

2.3.4.4 Haller Social Avoidance

The design of the testing apparatus followed Haller and Bakos (2002) (or see Appendix 1). At the time of testing, both the stimulus rat and the test rat were habituated

to their chambers for three minutes with the guillotine door closed. After three minutes, the guillotine door was opened to allow the test rat access to the middle chamber. The test rat could move freely in these two chambers. The test rat in the middle chamber could view and sniff the stimulus rat, but could not socially interact with it. After five minutes, the test rat was removed by a gloved hand and put back into its home cage. The apparatus had a clear plastic roof to allow videotaping of behavior of both rats from above for later analysis. Both the habituation and five minute test were recorded. The apparatus was cleaned with a 70% alcohol solution and wiped dry between tests.

Measures of the test rat's behavior taken from videotape included time immobile in the start and middle boxes, time spent in the start and middle boxes, and time spent near the stimulus rat's Plexiglas wall. This area was a rat width (8 cm) wide and marked with tape. The test rat was considered near when all four feet were within the taped region. Latency to first leave the start chamber and enter the middle chamber was taken as well as time spent interacting with the stimulus rat through the Plexiglas wall. Finally, we recorded the length of time the stimulus rats were immobile. It was thought that group differences in attention paid to, and time near, the stimulus rat might be affected by state of mobility of the stimulus rat. This measure was taken to see if behavior of the stimulus rat across groups was similar.

As previously mentioned, testing was done in cycles of 12 rats (2 for each group). Within each cycle a thirteenth rat was randomly assigned to be the stimulus rat in this test. This rat was used for this purpose only. Therefore, 10 stimulus rats were used, one

for each cycle of testing. The stimulus rats were handled for three days prior to the Haller social avoidance test.

2.3.4.5 Startle Testing

Unconditioned startle response to an acoustic stimulus was determined using a standard startle chamber (San Diego Instruments). The apparatus was fitted with a 20.3 cm long Plexiglas cylinder that was used to hold the animal, as well as a speaker for producing the sound bursts. Motion of the animal within the cylinder was detected via a piezoelectric transducer, which was positioned below the cylinder. Output of the transducer was led to a computer for sampling.

Prior to startle testing, animals were acclimatised to the apparatus for five minutes with a background white noise level of 60 db. Immediately thereafter, testing began. Rats were given 20 trials (1/min) of 20 msec bursts of 120 db of white noise rising out of a background of 60 db. A computer attached to the apparatus recorded 20 samples of transducer output. Samples included a 20 msec baseline and 250 msec sample after onset of the noise burst. Average transducer output just prior to the noise burst was saved as a baseline (VStart). In addition, the computer found the peak startle amplitude within each of the samples (Vmax) and this value was also saved for later analysis. Peak startle amplitude was expressed as Vmax-VStart for analysis. At the end of the startle session the rats were returned to their home cages. The apparatus was washed between rats.

2.3.5 Statistical Analysis

Behavioral measures were analyzed by one-way analysis of variance (ANOVA) examining the group differences for the six groups. Comparisons between the Handled control group and all cat exposed groups were done using planned comparison t-tests. The comparisons between all cat exposed groups were done using the Tukey-Kramer multiple comparison tests ($p<.05$). When data did not meet test assumptions of normality, non-parametric Kruskal-Wallis analysis of variance on medians was used. In this case, median contrasts were done using the Kruskal-Wallis Z test for multiple comparisons. For all measures, the predator stressed only and predator stressed plus vehicle groups did not differ. Therefore, these two groups were combined for subsequent analyses.

2.3.6 Ethical Approval

The research methods used in this experiment were reviewed for compliance with the guidelines of the Canadian Council on Animal Care (CCAC), and approved by the Institutional Animal Care Committee of Memorial University.

2.4 Results

2.4.1 Effects of Predator Stress on Behavior in the Hole Board and Plus Maze

One way ANOVAs revealed no group differences in behavior in the hole board in activity (rears or time active) or in exploration (data not shown). Group Effects were observed in standard measures of open arm exploration and risk assessment in the EPM. Data from open arm exploration appear in Figure 2.1 [all $F(4,115) > 5.16$, $p<.001$].

Predator stress reduced open arm exploration (ratio time and entry) in all groups except for the 10 mg/kg CPP group [all $t(115) > 6.23$, $p<.01$]. The 10 mg/kg dose returned ratio entry to control levels, and tended to return ratio time to control levels. Ratio time means for the 10 mg/kg group were greater than other predator stressed groups [$t(115)=4.67$, $p<.01$], but were below controls [$t(115)=2.05$, $p<.043$].

Ratio Frequency Risk assessment data were analyzed with a non-parametric Kruskal-Wallis one-way ANOVA because the data were not normally distributed (Omnibus Normality Test = 16.18 $p<0.0004$). There was a significant Group Effect [$H(4)=9.95$, $p<.041$, Figure 2.2]. Predator stress reduced risk assessment in all stressed groups except the 10 mg/kg CPP group (Kruskal-Wallis multiple Z test, all $Z > 2.31$, $p<.05$). Risk assessment of these rats fell between that of other stressed rats and handled controls.

Predator stress reduced the ethological measure of unprotected head dips, and CPP was without effect on this reduction at any dose [$F(4,115)=11.20$, $p<.001$; all $t(115)>4.05$, $p<.001$, Figure 2.2]. The predator stress effects on unprotected head dips are consistent with previous studies (Adamec, 2001; Adamec et al., 2001). Interestingly, there is a dissociation between unprotected head dips and open arm exploration with respect to effects of CPP. Often, changes in these two behaviors go together (Adamec, 2001; Adamec et al., 2003).

2.4.2 Effects of Predator Stress on Behavior in the Light/Dark Box

There was one Group effect on behaviors measured in the Light/Dark Box (Figure 2.3). Predator stress reduced numbers of entries into the lighted chamber, and 10 mg/kg CPP prevented this reduction, raising entries to control levels [$F(4,115)=2.99$, $p<.05$; all $t(115)>2.02$, $p<.045$].

2.4.3 Effects of Predator Stress on Behavior in the Social Interaction Test

Group Effects were found for one measure of social interaction, withdrawals from partner [$F(4,115)=2.51$, $p<.05$, Figure 2.4]. Predator stress increased withdrawals and CPP was without effect on this increase at any dose [all $t(115)>2.55$, $p<.02$]. Predator stress tended to decrease time spent in social interaction as well [$F(4,115)=2.32$, $p<.062$, Figure 2.4]. Because of the trend, planned mean contrasts on time spent in social interaction were carried out using Bonferroni protected t tests. Predator stress reduced time spent in social interaction, and CPP was without effect on this decrease at any dose [all $t(115)>2.54$, Bonferroni protected t tests, $p<.05$].

2.4.4 Effects of Predator Stress on Acoustic Startle

2.4.4.1 Effects on Peak Startle Amplitude

A two-way ANOVA assessed Group and Startle trial effects (with repeated measures on startle trial) for peak startle amplitude data. For this analysis, the twenty trials were collapsed into 10 blocks of 2 trials each. There was a Trial Block Effect [$F(9,1035)=20.95$, $p<.001$] and a slight trend toward a Group x Trial Block Effect

[F(36,1035)=1.21, p<.19]. Tukey Kramer tests on the Trial Block Effect revealed a habituation like decline in peak startle amplitude taking place over the first three trial blocks (p<.05, Block 1>2; Block 1>Blocks 3-10; Block 2 >Blocks 8-10; Blocks 3-10 are equal Figure 2.5, bottom panel).

2.4.4.2 Effects on Habituation of Startle

Predator stress has been shown to prolong habituation to startle (Adamec, 1997). For this reason, and given the slight trend toward an interaction, habituation to startle in the different groups was determined and compared. Exponential decline functions of the form:

$$y = y_0 + ae^{-b/\tau}$$

were fit to the peak startle amplitude mean data from each group using Jandel Table Curve V4.0. In the equation, y and y_0 are peak startle amplitude, a is a constant, e is the base of the natural logarithm, b is trial block and τ is the trial constant, or the number of trial blocks to decline to 37% of the maximal peak startle amplitude. Data were smoothed to improve fit. A Fast Fourier Transform (FFT) smoothing function provided in the program (20% FFT smooth) was applied to means from each group to improve fit. Care was taken to ensure the smoothing did not distort the data (see Figure 2.5). All fits were good [coefficients of determination range: .885 to .994; all Fit F(2,9)>27.0, p<.001; t(9)>2.82, p<.02 for all t tests of difference from zero of τ]. The estimate of τ included a standard error of estimate. These standard errors were used to perform t tests between the

trial block constants of the different groups of rats. The multiple comparisons between τ values estimated for each group were done using Bonferroni protected t tests (Figure 2.5).

As has been reported in the past (Adamec, 1997), predator stress decreased the habituation rate or increased trial block constant. This increase was blocked by the 1.0 and 10 mg/kg doses of CPP but not by the 0.1 mg/kg of CPP [Figure 2.5, all $t(18)>3.28$, $p<.05$ Bonferroni protected t tests].

2.4.5 Effects of Predator Stress on Behavior in the Haller Social Avoidance Test

There were no group differences on any measure in this test. Nor did the behavior of the stimulus rat (time immobile) differ among the groups. Therefore, neither predator stress nor CPP had any lasting effects on behavior in this test.

2.4.6 The Predator Stress Experience

To ensure group differences were not due to differences in the behavior of the cats used, all measures of cat and rat behavior were analyzed for Group Effects among the cat exposed groups of rats. There were no group differences with respect to any cat or rat behavior measured in the cat test situation.

2.4.7 Body Weight

Groups did not differ with respect to body weight at the beginning of the experiment, at treatment time, or at behavioral testing times (data not shown). These data are consistent with previous findings (Adamec, 1997; Adamec & Shallow, 1993).

2.5 Discussion

There were two purposes to this study. First, replicate past findings of the behavioral effects of CPP given prior to predator stress and extend those findings to doses other than the 10 mg/kg dose used originally (Adamec et al., 1999a). Second, broaden the battery of behavioral tests to determine whether predator stress-induced changes in behaviors, other than those originally investigated, are also NMDA receptor-dependent.

2.5.1 Behaviors Unchanged by Predator Stress

2.5.1.1 Anxioselectivity of Predator Stress-Induced Changes in ALB

As reported repeatedly in the past (Adamec, 1997; Adamec, 2001; Adamec et al., 2001; Adamec & Shallow, 1993; Adamec, Shallow, & Budgell, 1997) changes in ALB in the EPM cannot be accounted for by changes in exploratory tendency or activity. Measures of activity and exploratory behavior in the hole board or EPM were unaffected by predator stress or CPP. Moreover, neither predator stress nor CPP affected time spent in the center of the hole board or thigmotaxis (time spent near the wall). The latter measure is sometimes used as a measure of rodent anxiety. Clearly, it does not vary in the same way as open arm exploration in the EPM, which is our primary measure of rodent ALB. A lack of effect of predator stress on thigmotaxis measure is commonly observed in this laboratory.

2.5.1.2 The Haller Social Avoidance Test

Haller reported lasting effects of social defeat and shock on exploration of the larger chamber containing a strange stimulus rat (Haller & Bakos, 2002; Haller et al., 2003). In our study, however, there was no evidence that predator stress produced long-lasting social avoidance as measured in the Haller social avoidance test. There are several possible explanations for this result. First, the test is not sensitive to the effects of predator stress, so it may be stressor specific. Second, Haller reports effects lasting more than five days and less than 10 days. Our rats were tested eight days after stress. The effects of one predator encounter may have been insufficient to produce effects lasting eight days. Tests before eight days after predator stress are needed to determine whether this explanation is reasonable. Finally, the Haller test took place just after the social interaction test. Carry over effects from a direct social encounter prior to the Haller test may have interfered with a predator stress effect. This is an empirical question, requiring testing in the Haller box alone on a given day. However, test order effects are not likely a factor in our other results because previous studies using this battery (except for the Haller test) have counterbalanced order of testing with similar findings of effects of predator stress (Adamec et al., 2003).

2.5.2 Startle Amplitude

Previous work indicates that predator stress increases peak startle amplitude (Adamec, 1997) and this facilitation is dependent on NMDA receptors in the amygdala (Adamec et al., 1999b). In the current study, however, there were no effects of predator

stress on startle amplitude. Nevertheless, a failure to find effects of predator stress on startle amplitude is not without precedent in this laboratory. We have recently found (in preparation) that more robust and reliable increases in startle amplitude are achieved with a 10 minute cat exposure, rather than the five minute exposure used here.

2.5.3 NMDA Dependence of Predator Stress-Induced Behavioral Change

The present study replicates previous work showing that competitive block of NMDA receptors with 10 mg/kg of CPP prior to predator stress prevents changes in EPM behavior (ratio time, ratio entry, risk assessment) (Adamec et al., 1999a). The present study extends these findings to include the effects of a wider range of CPP dosages and the use of a more extensive battery of behavioral tests. The results show that a dose of 10 mg/kg, and not 0.1 or 1.0 mg/kg, of CPP is necessary to block changes in most behaviors caused by predator stress. The one exception is prolongation of startle habituation by predator stress, which is blocked at both the 1.0 and 10 mg/kg doses of CPP.

Inclusion of a wider variety of behavioral tests broadens the number of predator stress-induced behavioral changes that appear to be NMDA receptor-dependent. Entries into the lighted chamber of the light/dark box and startle habituation are two such behaviors, in that predator stress effects are blocked by CPP. Nevertheless, predator stress-induced changes in some behaviors are likely not NMDA receptor-dependent. For example, stress-induced decreases in time spent in social interaction, stress-induced increases in withdrawals from partner in social interaction and reduced unprotected head dips in the EPM were unaffected by CPP. It cannot be stated with certainty that changes

in these behaviors are not dependent on NMDA receptors, since a higher dose of CPP might be required. Whether or not a higher dose is required is unknown.

These observations are consistent with two conclusions. First, the selective effects of CPP argue against the idea that possible psychotomimetic effects of the drug at the time of predator stress may have interfered with the perception of the experience. If that were the case, there should be no effect of predator stress on social interaction or on unprotected head dips in the 10 mg/kg CPP group. Moreover, rats given any dose of CPP responded as defensively to the cat as rats not given CPP. Second, the lack of effect of CPP on social interaction is consistent with the view that separable neural substrates mediate stressor-induced changes in different behaviors. This view arises in part from factor analyses which have shown that social interaction, light/dark box and plus maze measures load on independent factors (Adamec et al., 2001; Adamec et al., 2003).

In the factor analysis just mentioned, unprotected head dips loaded on the EPM anxiety factor (involving ratio time and entry). If shared factor loading implies shared neural substrates, one would have expected CPP to block effects of predator stress on unprotected head dips, which was not the case in the present study. Rather, the present data suggest substrates mediating changes in open arm exploration and unprotected head dips differ, at least in the mechanism of initiation of long-lasting change. The factor loadings seen in previous studies may reflect a behavioral rather than a neural substrate association. In past studies, covarying open arm exploration from predator stress effects on unprotected head dips usually eliminated them (Adamec et al., 2003). This finding, in combination with the present results, suggests loading of unprotected head dips and open

arm exploration on the same factor reflects more the creation of an opportunity to perform the behavior by open arm exploration than a shared neural substrate.

Though predator stress potentiation of startle was not seen in the present study, we did replicate the predator stress-induced increase in the trial constant (τ) measure of habituation reported previously (Adamec, 1997). These results are particularly important for two reasons. First, the replication strengthens the parallels between PTSD and this preclinical model, since this kind of slowing of habituation to startle is observed in PTSD sufferers (Shalev, Orr, Peri, Schreiber, & Pitman, 1992). Second, it is a new finding that predator stress-induced delay in habituation appears to be NMDA receptor-dependent. Moreover, block of the habituation effect is achieved at lower doses of CPP than block of the effect of predator stress on EPM ALB. Thus, there appears to be a greater sensitivity to CPP in systems mediating changes in startle habituation. These findings are consistent with factor analyses showing startle and EPM ALB load on separate factors and, therefore, may be under the control of separate neural substrates (Adamec et al., 2003).

Taken in the context of past work, these findings are also consistent with the view that separate neural circuits mediate predator stress effects on startle amplitude and startle habituation. In the present study, there was no effect of predator stress on startle amplitude, but there was a prolongation of habituation of startle by predator stress, which was blocked by CPP. Therefore, both habituation and startle amplitude likely involve NMDA receptors, since NMDA receptor block also prevents predator stress increases in startle amplitude when they occur (Adamec et al., 1999b). It is likely that different neural

circuits, changed by stress through NMDA receptor action, mediate these two aspects of the startle response.

The identity of those circuits is not known; however, there are some clues. For instance, previous work implicates NMDA receptor-mediated LTP of amygdala efferent transmission (Adamec et al., 1999b; Adamec et al., 2003) in increases in startle amplitude. In contrast, habituation effects of predator stress could be mediated by NMDA receptor-dependent interference in synaptic depression elsewhere in the brain. This idea arises from studies suggesting homosynaptic depression in brain stem startle pathways mediates short-term startle habituation (Weber, Schnitzler, & Schmid, 2002).

2.5.4 Conclusions

This study replicates and extends past work on the effects of predator stress on behavior and the role of NMDA receptors in those effects. Many of the effects of predator stress on behavior appear to be NMDA receptor-dependent. However, predator stress effects on social interaction may not be NMDA receptor-dependent. In addition, predator stress does not appear to lastingly affect social avoidance. These findings add to a body of evidence that suggests a syndrome of behavioral changes follow predator stress. Moreover, these behavioral changes likely depend on changes in separable neural substrates.

Figure Captions

Figure 2.1. Plotted over experimental groups are mean + SEM of measures of open arm exploration in the plus maze (ratio entry and ratio time). For each measure, means marked with the same letter do not differ, but differ from means marked differently.

Figure 2.2. In the upper panel plotted over experimental groups are median ratio frequency risk assessment in the plus maze. Medians marked with the same letter do not differ, but differ from medians marked differently. Medians marked with the two letters fall between medians marked with those letters. Plotted in the lower panel are mean + SEM of unprotected head dips in the elevated plus maze over experimental groups. Means marked with the same letter do not differ, but differ from means marked differently.

Figure 2.3. Plotted for each experimental group are mean + SEM of entries into the lighted chamber in the light/dark box test. Means marked with the same letter do not differ, but differ from means marked differently.

Figure 2.4. Plotted over experimental groups are mean + SEM of social interaction test measures of number of withdrawals from test partner and time spent in social interaction (sec). Means marked with the same letter do not differ, but differ from means marked differently.

Figure 2.5. Plotted in the upper panel are mean + SEM of block constant values, τ, estimated from fits of declining exponential functions fit to mean peak startle amplitude over blocks of 2 trials for each experimental group. Values of τ marked with the same letter do not differ, but differ from values marked with a different letter. The lower panel shows an example fit (solid line) to an FFT smoothed (20%) function (dashed line) of the means of peak startle amplitude change over blocks of two trials (solid line with filled circles) over all rats.

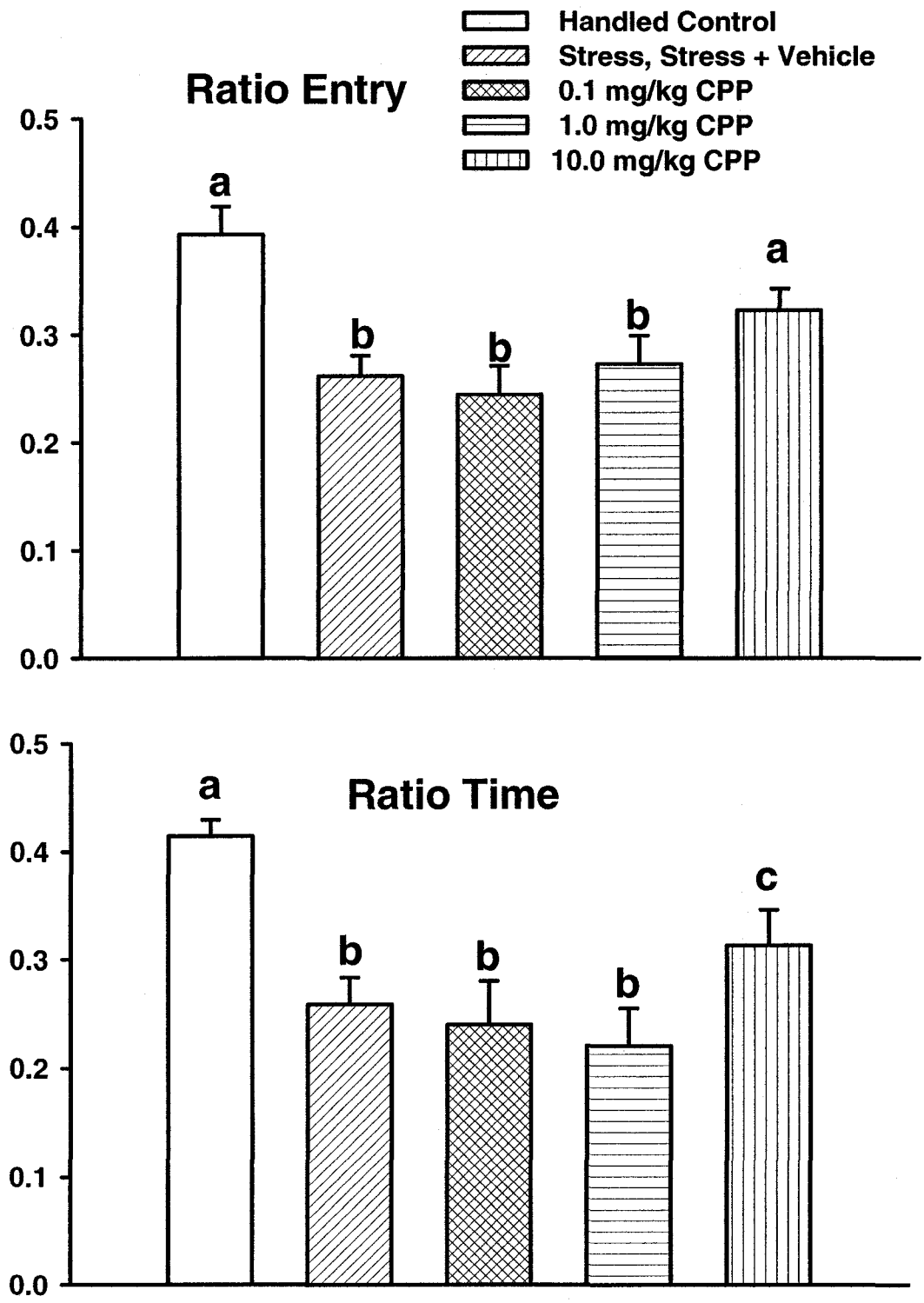

Handled Control
Stress, Stress + Vehicle
0.1 mg/kg CPP
1.0 mg/kg CPP
10.0 mg/kg CPP
Ratio Entry
0.5
0.4
0.3
0.2
0.1
0.0
a
b
b
b
a
Ratio Time
0.5
0.4
0.3
0.2
0.1
0.0
a
b
b
b
c

Figure 2.1

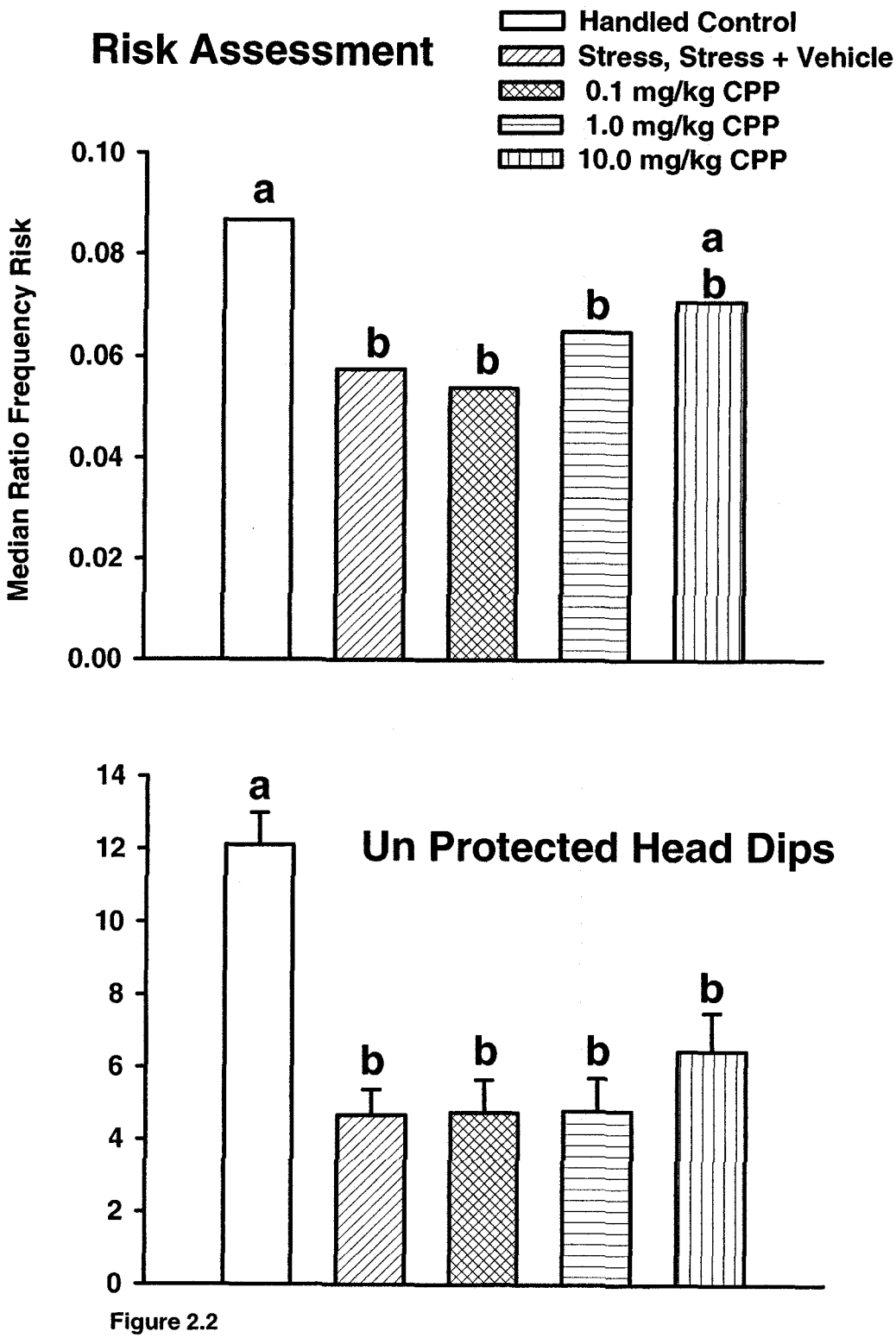

Risk Assessment
Handled Control
Stress, Stress + Vehicle
0.1 mg/kg CPP
1.0 mg/kg CPP
10.0 mg/kg CPP
Median Ratio Frequency Risk
0.10
0.08
0.06
0.04
0.02
0.00
a
b
b
b
a
b
Un Protected Head Dips
14
12
10
8
6
4
2
0
a
b
b
b
b

Figure 2.2

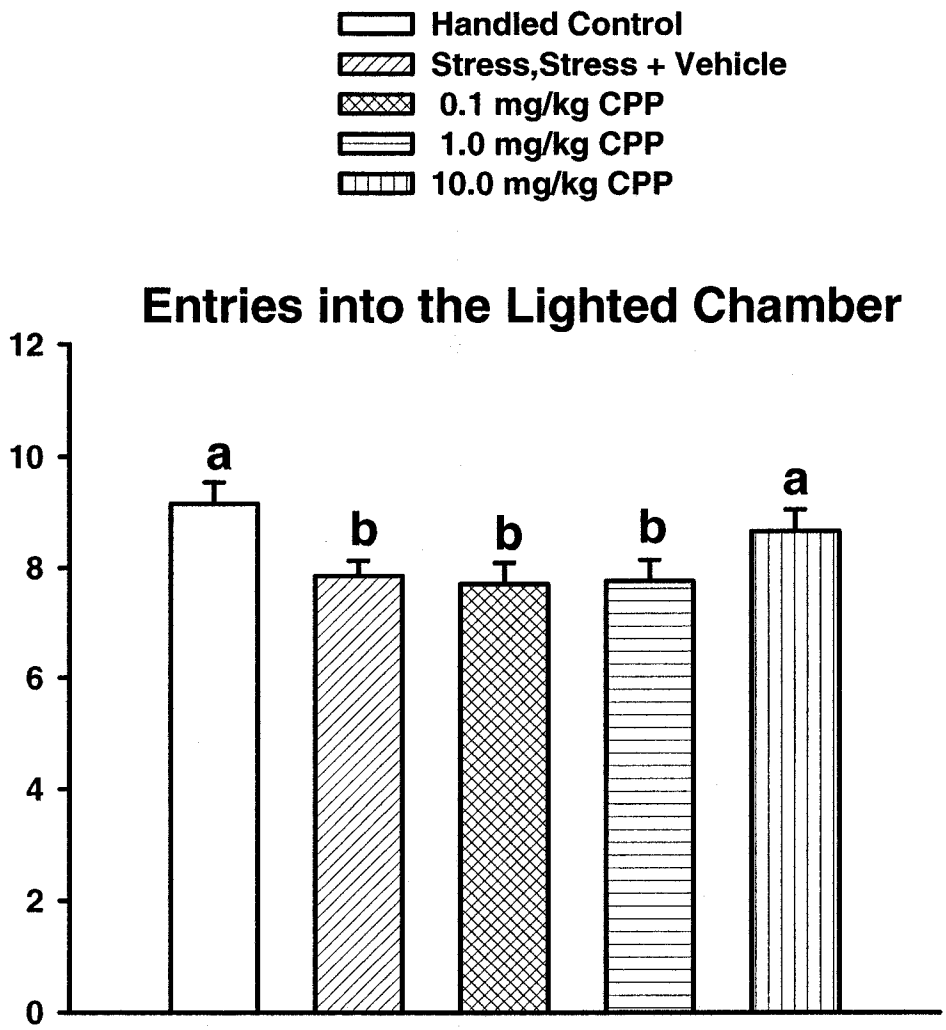
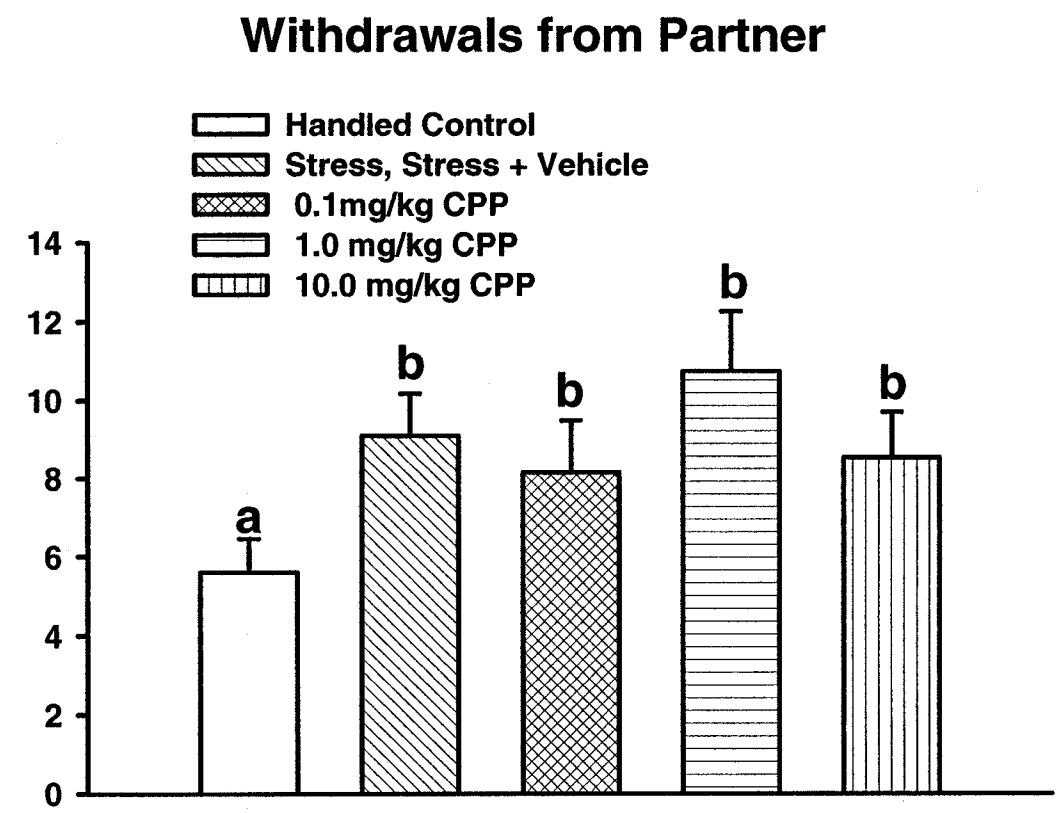

Handled Control
Stress,Stress + Vehicle
0.1 mg/kg CPP
1.0 mg/kg CPP
10.0 mg/kg CPP
Entries into the Lighted Chamber
12
10
8
6
4
2
0
a
b
b
b
a

Figure 2.3


Withdrawals from Partner
Handled Control
Stress, Stress + Vehicle
0.1mg/kg CPP
1.0 mg/kg CPP
10.0 mg/kg CPP
14
12
10
8
6
4
2
0
a
b
b
b
b

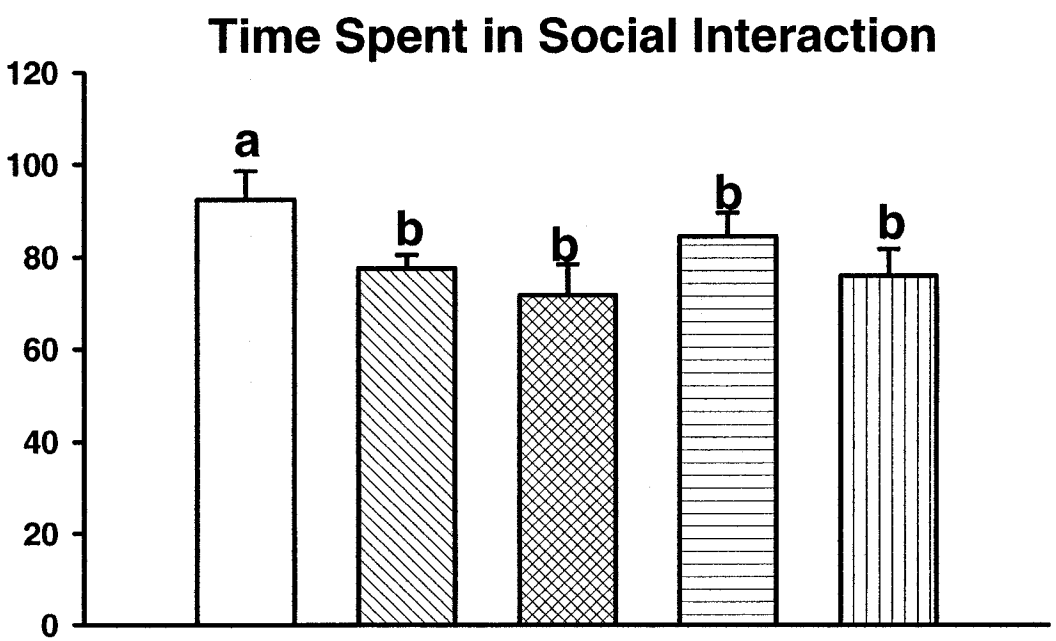

Time Spent in Social Interaction
120
100
80
60
40
20
0
a
b
b
b
b

Figure 2.4

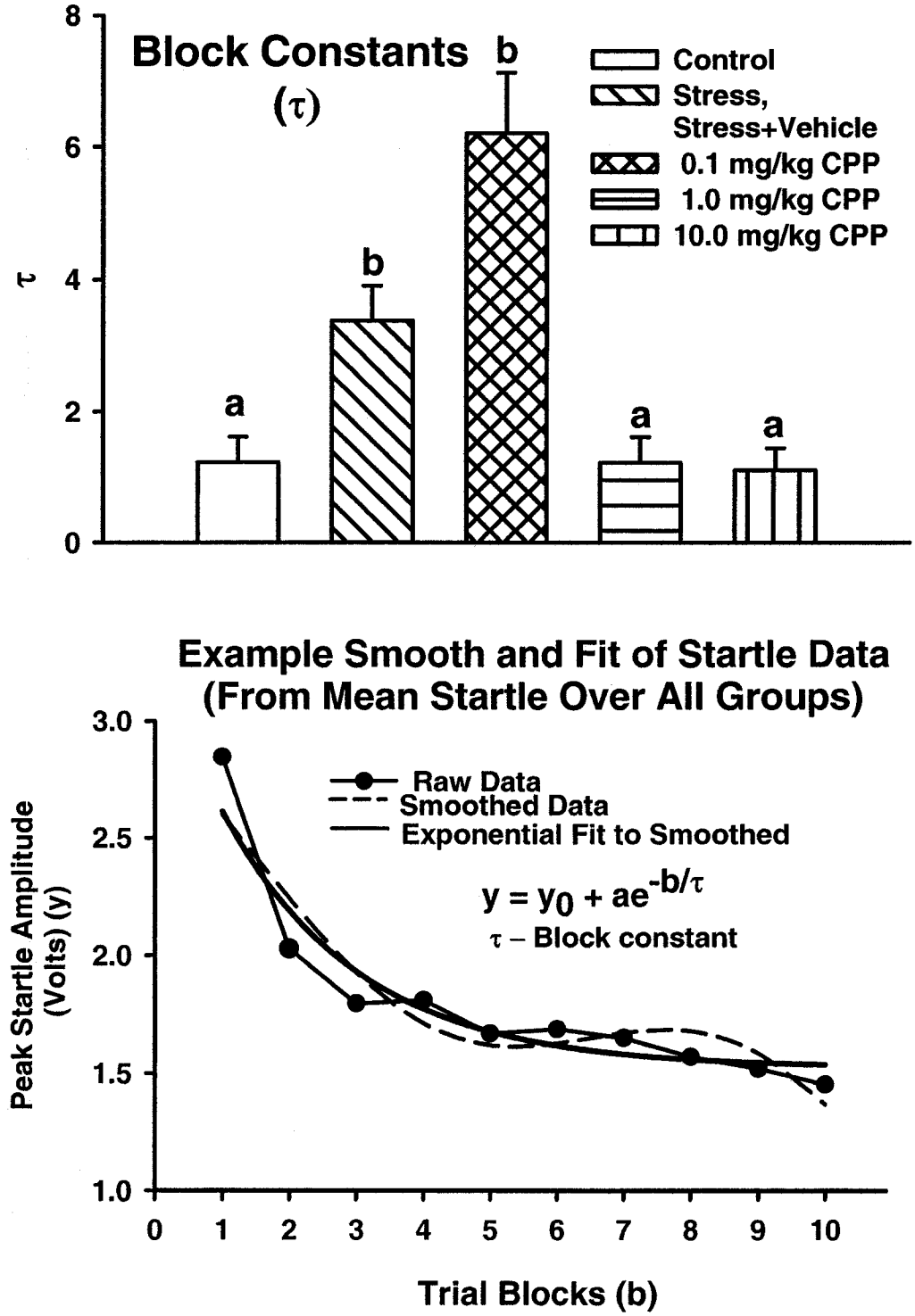

Block Constants
(τ)
τ
8
6
4
2
0
a
b
b
a
a
Control
Stress,
Stress+Vehicle
0.1 mg/kg CPP
1.0 mg/kg CPP
10.0 mg/kg CPP
Example Smooth and Fit of Startle Data
(From Mean Startle Over All Groups)
Peak Startle Amplitude
(Volts) (y)
3.0
2.5
2.0
1.5
1.0
Raw Data
Smoothed Data
Exponential Fit to Smoothed
y = y0 + ae-b/τ
τ – Block constant
0
1
2
3
4
5
6
7
8
9
10
Trial Blocks (b)

Figure 2.5

CHAPTER 3:

The NMDA Receptor Antagonist CPP Blocks the Effects of Predator Stress on pCREB in Brain Regions Involved in Fearful and Anxious Behavior.

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Canada
A1B 3X9

3.1 Abstract

A five minute unprotected exposure to a cat produces long-lasting anxiogenic effects on behavior which are *N*-methyl-*D*-aspartate (NMDA) receptor-dependent (Adamec, Shallow & Budgell, 1999a; Blundell et al., 2005 - Chapter 2). Since phosphorylation of cyclic AMP response element binding protein (CREB) is regulated by NMDA receptors and pCREB-like-immunoreactivity (lir) is increased after predator stress, we examined the effects of CPP (3-(2- carboxypiperazin4-yl)propyl-l-phosphonic acid), an NMDA receptor antagonist, on predator stress-induced changes in pCREB-lir in brain areas implicated in fearful and anxious behavior including the amygdala, periqueductal gray (PAG), bed nucleus of the stria terminalis (BNST), anterior cingulate cortex (ACC), and dorsal medial hypothalamus (DMH). Results showed that CPP blocked the predator stress-induced increase in pCREB-lir in the right lateral PAG, blocked the predator stress-induced increase in pCREB-lir in several amygdala nuclei and reversed the predator stress induced suppression of pCREB-lir in the BNST. Importantly, at least in the amygdala and PAG, the pattern of pCREB-lir was hemisphere- and AP plane-dependent. Our results suggest that several amygdala nuclei, the PAG, and the BNST, where predator-stress changes pCREB-lir in an NMDA receptor-dependent manner, are candidate areas of neuroplastic change contributing to lasting changes in anxiety-like behaviors.

3.2 Introduction

In recent years, there has been growing interest in the long-lasting changes in brain and behavior that occur after stressful events. This interest has been heightened due to the fact that fearful events may cause psychopathologies (Harvey & Rapee, 2002; Yehuda, 2002). In extreme cases, a single exposure to an aversive event may cause an individual to develop posttraumatic stress disorder (PTSD) (North, Nixon, Shariat, Mallonee, McMillen, Spitznagel, & Smith, 1999; Silver, Holman, McIntosh, Poulin, & Gil-Rivas, 2002). Recently, researchers have turned to animal models to investigate stress-precipitated psychopathologies (i.e., PTSD). Animal models are useful because they provide the opportunity to simulate a human condition in a controlled setting and they allow the disorder to be studied as it develops. Furthermore, pharmacological and other treatments that may be difficult to test in humans but can be easily evaluated in animals. Conditioned fear paradigms, behavior in unfamiliar situations that are fear or anxiety provoking, and more recently, predator stress, are all models used to understand the neurobiology of fearful events.

Predator stress involves the unprotected exposure of a rat to a cat (Adamec & Shallow, 1993). It has been argued that predator stress models aspects of PTSD for several reasons. First, this model has a high degree of ecological validity due to the natural threat posed by the predatory nature of the stressor. Second, duration of anxiety-like effects in rats after predator stress, as a ratio of life span, is comparable to the DSM IV duration of psychopathology required for a diagnosis of chronic PTSD in humans (Adamec, 1997; Adamec & Shallow, 1993, Adamec, Shallow & Budgell, 1999a). Third,

this model has neurobiological face validity in that right amygdala and hippocampal circuitry are implicated in behavioral changes produced by predator stress and these areas are consistent with brain areas thought to be involved in PTSD (Adamec, Blundell, Burton, *in press*). For example, brain imaging studies implicate the anterior temporal lobe (Shin, McNally, Kosslyn, Thompson, Rauch, Alpert, Metzger, Lasko, Orr, & Pitman, 1997) and in particular, hyperexcitability of the right amygdala in response to script-driven trauma reminders in PTSD patients (Rauch, Savage, Alpert, Fischman, Jenike, 1997; Rauch & Shin, 1997; Rauch, van der Kolk, Fisler, Alpert, Orr, Savage, Fischman, Jenike, & Pitman, 1996; Shin et al., 1997; Shin, McNally, Kosslyn, Thompson, Rauch, Alpert, Metzger, Lasko, Orr, Pitman, 1999). Fourth, parallel path analytic studies have been done using data from Vietnam veterans suffering from PTSD and predator stressed rodents to determine whether analogous relationships exist between instigating conditions and subsequent changes in affect (Adamec, 1997). In both humans and rodents, features of the stressor predict the level of anxiety. In the predator stress model, for example, the more cat bites received, the higher the level of anxiety measured a week later in the rat. Finally, similar lasting changes in startability and habituation of startle are seen in both predator stressed rats and humans with PTSD (Adamec, Kent, Anisman, Shallow & Merali, 1998; Adamec, 1997).

Predator stress is both fear provoking and stressful (Adamec et al., 1998; Blanchard, Nikulina, Sakai, McKittrick, McEwen, & Blanchard, 1998; Dielenberg, Carrive, & McGregor, 2001a; McGregor, Schrama, Ambermoon, & Dielenberg, 2002). In particular, exposure to a cat produces long-lasting increases in rat anxiety-like

behavior (ALB) (Adamec & Shallow, 1993; Adamec et al., 2003; Blundell, Adamec, & Burton, 2005 - Chapter 2) with some behavioral changes lasting at least three weeks after cat exposure (Adamec, 1997; Adamec & Shallow, 1993). Behavioral effects of predator stress have been evaluated in a number of tests including hole board, elevated plus maze (EPM), unconditioned acoustic startle, light/dark box and social interaction.

In addition to the behavioral changes, amygdala efferent and afferent neural transmission is altered after predator stress. Specifically, predator stress causes a long-lasting potentiation in neural transmission from the amygdala (central nucleus-Ce) to the lateral column of the periaqueductal gray (lPAG) and from the hippocampus via the ventral angular bundle (VAB) to the basolateral amygdala (BLa) (Adamec, Blundell, & Collins, 2001; Adamec et al., 2003; Adamec, Blundell & Burton, 2005a). This suggests a long term potentiation (LTP)-like change in amygdala afferent and efferent transmission following predator stress. More importantly, this enhanced potentiation is blocked by an NMDA receptor antagonist (Adamec, Blundell, & Burton, 2005b).

Amygdala afferent and efferent LTP-like changes are highly predictive of severity of change in ALB following predator stress (Adamec et al., 2003). Moreover, LTP-like changes in these pathways have been proposed as a mechanism mediating stress-induced changes in ALB. Furthermore, like stress-induced LTP-like changes, alterations in ALB following predator stress are also NMDA receptor-dependent. For instance, systemic administration of both competitive and non-competitive NMDA receptor antagonists before, but not after, predator stress prevent lasting changes in ALB (Adamec et al., 1999a; Blundell et al., 2005 - Chapter 2). Also, local NMDA receptor block in the

amygdala prevents predator stress-induced increases in ALB (Adamec, Burton, Shallow, & Budgell, 1999b). In addition to the amygdala, NMDA receptor block produces anxiolytic-like effects when microinjected into the dorsolateral PAG (Guimaraes, Carobrez, de Aguiar, & Graeff, 1991; Molchanov & Guimaraes, 2002). The PAG is also implicated in rodent ALB (Brandao, Anseloni, Pandossio, De Araujo, & Castilho, 1999) and is activated by predator stress (Canteras & Goto, 1999).

Changes in ALB and amygdala neural transmission are accompanied by changes in phosphorylated cAMP response element binding protein (pCREB) (Deisseroth, Bito, & Tsien, 1996). Specifically, pCREB-like-immunoreactivity (lir) is elevated in the basomedial (BM), BLa, Ce and lateral (La) amygdala after predator stress compared to control rats (Adamec et al., *in press*). This is consistent with research that has found elevated pCREB-lir in the amygdala after forced swimming (Bilang-Bleuel, Rech, De Carli, Holsboer & Reul, 2002; Shen, Tsimberg, Salvador & Meller, 2004), fear-conditioning in mice (Davies, Tsui, Flannery, Li, DeLorey, & Hoffman, 2004), retrieval of a cued-fear memory (Hall, Thomas, & Everitt, 2001), and electric shock (Stanciu, Radulovic & Spiess, 2001). In addition to the amygdala, predator stress increases pCREB-lir in the right lateral column of the PAG (lPAG) (Adamec et al., 2003).

As discussed above, an NMDA receptor antagonist given prior to predator stress blocks increases in ALB and potentiation of amygdala afferent and efferent neural transmission. Since phosphorylation of CREB is regulated by NMDA receptors (Segal & Murphy, 1998) and pCREB-lir is increased after predator stress (Adamec et al., 2001;

Adamec et al., 2003; Adamec et al., *in press*), the question remains whether NMDA a receptor antagonist can block the predator stress-induced enhancement of pCREB-lir.

Thus, in the present study, an NMDA receptor antagonist (CPP (3-(2-carboxypiperazin4-yl)propyl-l-phosphonic acid)) was systemically injected 30 minutes prior to predator stress. CPP was chosen to match the antagonist used in previous studies of the effects of NMDA receptor block on brain and behavior in predator stressed rats discussed above (Adamec et al., 2005b; Adamec et al., 1999a; Adamec et al., 1999b; Blundell et al., 2005 - Chapter 2). We examined pCREB-lir in the amygdala, the PAG, the bed nucleus of the stria terminalis (BNST), the anterior cingulate cortex (ACC), and the dorsal medial hypothalamus (DMH). The BNST was included because of its importance in unconditioned fear responses to predator odor and a brightly-lit environment (Fendt, Endres, & Apfelbach, 2003; Walker & Davis, 1997). The ACC has been implicated in emotional processing (Garavan, Pankiewicz, Bloom, Cho, Sperry, Ross, Salmeron, Risinger, Kelley, & Stein, 2000) as lesions in this area have produced a wide variety of symptoms, including apathy, inattention, autonomic dysregulation, emotional instability and akinetic mutism (Bush, Luu, & Posner, 2000; Devinsky, Morrell, & Vogt, 1995). Moreover, dysfunction and volumetric reductions in the ACC have been associated with PTSD (Rauch, Shin, Segal, Pitman, Carson, McMullin, Whalen, Makris, 2003; Shin, Whalen, Pitman, Bush, Macklin, Lasko, Orr, McInerney, & Rauch, 2001). Also, rodent ACC has been implicated in appetitive and aversive stimulus-reinforcer learning. For example, lesions of the ACC impair acquisition of the avoidance response (Gabriel, 1993; Gabriel, Kubota, Poremba, & Kang, 1991). Finally,

the DMH has been shown to play an important role in stress-related signals to the hypothalamo-pituitary-adrenal (HPA) axis (DiMicco, Samuels, Zaretskaia, & Zaretsky, 2002; Herman, Prewitt, & Cullinan, 1996; Herman & Cullinan, 1997) and is involved in regulating rodent ALB (Shekhar, 1993; Shekhar, Katner, Sajdyk, & Kohl, 2002).

3.3 Methods

3.3.1 Animals

Twenty-four male hooded Long Evans rats (Rattus norvegicus) The Charles River Breeding Farms, Quebec, were used in this experiment. All rats were housed alone in clear polycarbonate cages measuring 46 cm x 24 cm x 20 cm for at least four days before testing began. Rats were given food and water *ad lib* and they were exposed to a 12-hour light/dark cycle with lights on at 7:00 AM. Rats weighed approximately 200 g on arrival and between 230 g and 282 g on the day of testing. All rats were handled in the same room as their home cages one minute a day for three days prior to testing. Handling involved picking up the rat with a gloved hand and gently holding it on the forearm. A minimal amount of pressure was used if the rat attempted to escape and the grip was released as soon as the rat became still.

3.3.2 Groups

The rats were randomly assigned to one of four groups (n=6 rats per group). The four groups were; predator stressed [exposed to a cat (E)], vehicle handled control (VC), vehicle (0.5 ml of saline ip) plus cat exposed (VE), and CPP (10 mg/kg in 0.5 ml of

saline ip) plus cat exposure (ECPP). Until the testing day, all rats were treated the same. Care was taken to ensure that the rooms used to hold the rats were void of cat odor. The cat was only permitted in the exposure room.

3.3.3 Testing

On the day of testing, a multiple of four animals was euthanized (ensuring one animal from each group was tested). The order of testing was counterbalanced for each set of four rats. In addition, testing began at 10:00 AM and ended at 4:00 PM with care taken that one animal from each group was tested at different times throughout the day. On this day, all animals were weighed immediately before testing began.

3.3.3.1 Cat Exposure

Predator stressed groups (E, VE, ECPP) were exposed to a cat on the day of testing. Cat exposures occurred in a large wooden room with carpet on the floor. For more details on the room, see Adamec and Shallow (1993). Four different cats were used in this experiment and all cats were counterbalanced across all three predator-exposed groups. The cat was placed in the room at least one hour before testing. Thirty minutes prior to the cat exposure, rats in the ECPP and VE groups were injected with either CPP or vehicle. Immediately prior to cat exposure, a rat was placed in a wooden enclosure and transported to the exposure room. The rat enclosure fits into a small opening at the floor of the exposure room. This small door was opened and the rat was gently forced, via a sliding platform inside the enclosure, to enter the room. The door was then closed

and testing began. This method allowed the introduction of the rat into the room without handling. The five minute exposure was videotaped to capture the activity of both the cat and the rat. Cat response to the rat ranged from watching the rat at a distance, to approach and sniffing, with the occasional mild attack. Sometimes the cat pawed and bit a rat but did not physically injure it. All rats were examined for wounds after the cat encounters, and none were observed. After testing, the rat was placed back in its home cage (which had been moved to a dark, novel room) and left undisturbed for ten minutes. Importantly, only the cat exposed rats were placed in this room. At this time, the rat received an ip injection with an overdose of sodium pentobarbital (1 ml at a concentration of 65 mg/ml). Ten minutes later, the rat was checked for a reaction (if the rat still displayed a reflex, it was given a supplementary dose of 0.1 ml). If no reaction was observed, the rat was perfused with 200 ml of heparinized saline followed by 500 ml of paraformaldehyde. The timing of the perfusion was important because it has been shown that pCREB peaks between 20 and 25 minutes after exposure to stimuli (Silva, Kogan, Frankland, & Kida, 1998). The brain was removed, placed in a 20% sucrose solution overnight, and subsequently flash frozen in isopentane cooled by liquid nitrogen. The brain was left in a -70°C freezer until sectioning.

3.3.3.2 Treatment of Vehicle Controls

Rats in the vehicle control group (VC) did not come in contact with the cat, cat odors, or rats that had previously been exposed to cats. On the day of testing, rats in this group were weighed and then injected with saline. Thirty minutes after injection, the rat

was moved to a new room (room was void of cat odor) and handled for one minute. After handling, the rat was placed back in its home cage for 10 minutes in the dark. The rat was then injected with an overdose of sodium pentobarbital and treated in the same manner as the cat exposed groups.

3.3.4 Behavioral Measures Taken From Cat Exposures

Behavior of the rat and the cat was videotaped and later analyzed to provide a measure of the cat exposure experience among the groups. Cat behaviors scored were; frequencies of approaches to the rat, latency to approach and time spent near the rat, latency to sniff and time spent sniffing the rat, latency to bite and frequency of bites and pawing of the rat. The floor of the exposure room was divided into one foot squares with masking tape. Time spent near the rat was scored when the cat was within one foot of the rat.

The responses of the rat were also recorded and analyzed. Frequencies of active, passive and escape defensive responses were measured. Active defense was scored in several ways: rat initiated approaches to the cat (active approach); rat bites; upright postures with or without pushing the cat with a forepaw; and rat vocalizations. Passive defense was scored when the rat became immobile for 1 sec or more when the cat approached or remained near. Escape was scored whenever there was a rapid movement of the rat away from the cat when the cat approached or was near.

3.3.5 Immunocytochemistry (ICC)

Forty μm coronal sections were cut in a cryostat and all sections were cut using the same cryostat. Twelve sections were taken from 5.8 mm to 6.8 mm posterior to bregma to capture the PAG; eight sections were taken from 1.8 mm to 3.6 mm posterior to bregma to capture the amygdala and DMH. In addition, four sections were taken from 0.26 mm to 0.92 mm posterior to bregma to capture the BNST and ACC (Paxinos & Watson, 1982). Anterior-Posterior (AP) plane location was determined by counting sections from the decussation of the anterior commissure (AP 0.26 mm posterior to bregma, Paxinos & Watson, 1982) to the desired AP plane. This permitted estimation of AP plane position to the nearest 40 μm during cutting. A multiple of four brains, one brain from each group, was cut at the same time and brains were processed using six sections per well (sections from only one brain). Sections were washed with phosphate buffered saline (PBS), saturated with normal goat serum (NGS) and Triton X-100 in PBS, washed again in PBS, then incubated at -4°C for either 24 or 48 hours (re-used antibody) in the primary antibody (rabbit anti-rat phosporylated CREB, 1/500 dilution, Upstate Biotech). Sections were washed with PBS, then incubated in the secondary biotinylated antibody (goat anti-rabbit) followed by the avidin-biotin complex (Vector ABC kit). For visualization, diaminobenzadine was used as the chromogen (Sigma tablet). Sections were then washed with PBS, mounted onto slides, dehydrated, and then cover slipped. For specific details on the pCREB staining protocol, see Appendix 2.

To control for non-specific staining, the ICC procedure described above was repeated without the primary antibody (Figure 3.1, top right photomicrograph). In

addition, to determine the specificity of the primary antibody, the primary antibody was saturated with pCREB prior to staining (Figure 3.1, bottom right photomicrograph). Both sections show little or no staining.

3.3.6 Densitometry Analysis

Stained sections were analyzed blind to group assignment using image analysis software (Jandel, MOKA software). Densitometry was used to quantify the data and hemispheres were measured separately.

The PAG was divided into ventral, dorsal and lateral areas to reflect the functional columnar organization described by Bandler, Carrive, and Depaulis (1991). This was done using the aqueduct of Sylvius as a guide. Horizontal lines were drawn from the top of the aqueduct to the outside edge of the PAG and from the bottom of the aqueduct to the outside edge of the PAG for both left and right sides. The top columns were considered dorsal PAG (dPAG), the middle columns were lateral PAG (lPAG) and the bottom columns were ventral PAG (vPAG). An example of pCREB staining in the PAG can be found in Figure 3.1 (top left photomicrograph).

The amygdala was divided into its nuclei: central (Ce), basolateral (BLa), lateral (La), basomedial (BM), ventral basolateral (BLv), medial (Me), anterior cortical (ACo), posterior cortical (PCo), and bed nucleus of the accessory olfactory tract (BAOT). Nuclear boundaries were determined with templates from different AP planes defining the nucleus from the rat atlas of Paxinos and Watson (Paxinos & Watson, 1982). A given brain section was assigned to the nearest atlas template. Straight lined shapes (i.e.,

square, rectangle, triangle, rhombus, etc.) were then created from each template for all nuclei in each AP plane in order to maximize coverage of the given nucleus. These shapes were then uniformly applied to all nuclei in each AP plane across all groups. Coordinates set by the templates were mapped onto the actual section, which corrected for tissue shrinkage. Right and left hemispheres were measured separately using the template shapes described above. An example of pCREB staining in the amygdala can be found in Figure 3.1 (bottom left photomicrograph).

The BNST was divided into lateral, medial and ventral within the AP range 0.26 mm to 0.40 mm posterior to bregma. The section that corresponded with AP 0.92 mm posterior to bregma was considered posterior BNST. Coordinates set by the templates were mapped onto the actual section for densitometry analyses, which corrected for tissue shrinkage. Again, right and left hemispheres were measured and analyzed separately. Densitometry measures of the DMH and the ACC were also taken in both right and left hemispheres using a similar methodology.

Stained sections were analyzed blind to group assignment using image analysis software (Jandel, MOKA software). For all brain areas, raw pCREB-lir densitometry data were converted to optical densitometry (OD) units. This was done by converting the raw densitometry data to OD units via a calibrated step wedge. An image of the calibrated step wedge was taken at the same time as section images for each rat. Exponential fits of raw transmission values (x) to calibrated OD values were done by computer (Table Curve, Jandel). All fits were good (all df adjusted $r^2>.9$, $p<.01$). The exponential was then used to interpolate and convert raw transmission values to OD

units. For the PAG (Adamec et al., 2003), BNST, and ACC, analyses were performed on the ratio of average OD values in a particular brain area to average OD values for the entire section. For the amygdala, analysis was performed on the ratio of average OD values of a standard 1 mm square sampled from the internal capsule in the hemisphere in which the particular brain area measure was taken (Adamec et al., *in press*). The same methodology was used to analyze the DMH.

Relative densitometric values were analyzed with three-way mixed ANOVAs examining Group x Hemisphere x Brain area, with repeated measures on Hemisphere and Brain area (i.e., amygdala nucleus). In the PAG, relative densitometric values were analyzed with three-way mixed ANOVA examining Group x Hemisphere x AP plane, with repeated measures on Hemisphere and AP plane separately for the dorsal and ventral columns. Right and left hemispheres were analyzed separately for the lPAG because Adamec et al. (2003) showed a right/left difference in pCREB-lir after predator stress. In the DMH and ACC, however, relative densitometric values were analyzed with two-way mixed ANOVAs examining Group x Hemisphere, with repeated measures on Hemisphere. Planned comparisons were done using t-tests and unplanned comparisons were done using Bonferroni protected t-tests and Tukey-Kramer multiple-comparison tests.

3.3.7 Stereology for the CPP Study

Previous research has shown predator stress increased pCREB-lir in the right lPAG and amygdala when measured densitometrically (Adamec et al., 2003; Adamec et

al., *in press*). However, when cells stained for pCREB were counted using stereological cell counting, the differences in pCREB-lir across groups were lost. These results suggest that the intensity of pCREB-lir per cell is elevated and not an increase in cell numbers stained for pCREB. To shed light on this issue, we divided and counted cells in the right lPAG in two categories: 1; cells stained completely black/dark and 2; all other stained cells. Since predator stress increases the density of pCREB staining, it was hypothesized that there would be more black/dark cells in the predator stressed groups (E and VE) than the control group (or ECPP group).

Stereological cell counting involved counting cells stained positive for pCREB-lir using Stereoinvestigator software. The optical fractionator method was employed using a 75 μm counting frame and 63x magnification in optical oil. We sampled 20 frames per lPAG for a given rat because it has been shown to yield low (<0.05) Schaeffer coefficients of error (CE) (Adamec et al., 2003; Adamec et al., *in press*). Tissue thickness was estimated stereologically. Mounted and cover-slipped tissue shrinkage of just over 50% was observed. Taking shrinkage into account and number of sections sampled (a total of 12) the software was used to estimate both the total volume of tissue which counts for each lPAG area and the total number of stained cells within that volume. Counts were done blind to group assignment.

3.3.8 Ethical Approval

The research methods used in this experiment were reviewed for compliance with the guidelines of the Canadian Council on Animal Care (CCAC), and approved by the Institutional Animal Care Committee of Memorial University.

3.4 Results

3.4.1 The Predator Stress Experience

To ensure group differences were not due to differences in the behavior of the cats used, all measures of cat and rat behavior were analyzed for Group Effects among the cat exposed groups of rats. There were no group differences with respect to any cat or rat behavior measured in the cat test situation (data not shown).

3.4.2 Amygdala pCREB Analysis

The amygdala was divided into three AP plane regions: the anterior (1.8 mm posterior to bregma), the middle range (2.12 mm to 2.68 mm posterior to bregma) and the more posterior range (greater than 2.68 mm posterior to bregma). This was done to compare with previous work that examined pCREB-lir after predator stress in the middle range only (Adamec et al., *in press*).

Relative optical density (OD) unit data were analyzed with a three-way mixed ANOVA assessing Group [cat exposed (E) or cat exposed plus vehicle (VE)], Hemisphere and Nucleus with repeated measures on Hemisphere and Nucleus separately for each of the three AP plane ranges. There were no Group effects or Group interactions

in the anterior and middle ranges, thus the two groups were combined to reduce complexity of further analyses [all $F<2.33$, $p>0.05$)], and are referred to as cat exposed combined (EC). For both these AP plane ranges, we can conclude that vehicle injection had no effect on pCREB-lir in predator stressed rats. However, in the more posterior AP plane range, there was a Group effect [$F(7,53)=2.75$, $p<0.05$)]. Therefore, the cat exposed (E) and cat exposed plus vehicle (VE) groups for the more posterior range differed and could not be combined.

For the anterior and mid AP plane range, relative OD unit data were analyzed with a three-way mixed ANOVA assessing Group (VC, EC, and ECPP), Hemisphere and Amygdala Nucleus with repeated measures on Hemisphere and Nucleus. In the posterior AP plane range, relative OD unit data were analyzed with a three-way mixed ANOVA assessing Group (VC, E, VE, and ECPP), Hemisphere and Amygdala Nucleus with repeated measures on Hemisphere and Nucleus.

3.4.2.1 Anterior AP Plane (1.8 mm posterior to bregma)

In the anterior AP plane range, there was a three-way Group x Nucleus x Side interaction [$F(10,90)=2.33$, $p<0.05$]. To determine the nature of the interaction, mean contrasts were made among the three groups (VC, EC, and ECPP) for each nucleus and in each hemisphere using t-tests. Details of the mean comparisons (t values and p values) can be found in Table 3.1a. Planned comparisons were done with t-tests and other comparisons with Bonferroni protected t-tests.

In the Ce, ACo, Me, BAOT in both hemispheres, predator stress increased pCREB-lir compared to controls and CPP blocked this predator stress-induced increase, returning ECPP levels to control (VC) (Table 3.1a, Figure 3.2).

The La and BLa showed the same pattern described above in the right hemisphere but not in the left hemisphere. In particular, predator stress increased pCREB-lir compared to controls and CPP blocked this increase in the right hemisphere (no difference between ECPP and VC groups; Table 3.1a, Figure 3.3). In the left hemisphere, however, there were no differences across groups (Table 3.1a, Figure 3.3).

Comparisons were also made between the same nuclei across hemispheres to assess hemispheric differences. Differences were found in the BAOT, BLa, and La (all $t(16)>2.21$, $p<0.05$; Figures 3.2, 3.3). In the BAOT, pCREB-lir was elevated in the left hemisphere in the VC and EC groups as compared to the right hemisphere and in the left BLa predator stressed rats showed less pCREB-lir than in the right BLa. Similarly, left La values differed from right La in the EC and in the ECPP groups (Figure 3.3).

3.4.2.2 Mid Range AP Plane (2.12 mm to 2.68 mm posterior to bregma)

There was a three-way Group x Nucleus x Side interaction [$F(14,133)=1.8$, $p<0.05$]. Planned comparisons were made among the three groups (VC, EC, ECPP) for each nucleus and in each hemisphere using t-tests, and other comparisons were made using Bonferroni protected t-tests. Mean comparisons (t values and p values) across groups can be found in Table 3.1b.

A similar pattern of pCREB-lir across groups was seen in the BM, BLa and BLv. Exposure to a cat increased pCREB-lir compared to controls in both hemispheres, replicating past findings (Adamec et al., *in press*). In the right hemisphere, CPP blocked this increase returning pCREB-lir to control levels (Table 3.1b, Figure 3.4). In the left hemisphere, however, CPP was without effect on the predator stress enhancement of pCREB-lir (BLv), or CPP actually potentiated the predator stress enhancement of pCREB-lir (BM, BLa; see Table 3.1b, Figure 3.4).

The Ce, La and Me showed a completely different pattern of pCREB-lir, as controls and predator stressed rats showed equal and less pCREB-lir than the ECPP group in the left hemisphere (Table 3.1b, Figure 3.5). In the right hemisphere, the La and Me showed similar pCREB patterns in that there were no differences across groups (Table 3.1b, Figure 3.5). In the Ce, however, pCREB-lir in the controls was the highest, lowest in the ECPP group, with predator stressed rats falling between the two (Table 3.1b, Figure 3.5).

The remaining two nuclei, the ACo and PCo, also showed different patterns of pCREB-lir across groups and hemispheres. In the ACo, exposure to a cat increased pCREB-lir compared to controls in both hemispheres (Table 3.1b, Figure 3.6). CPP tended to reduce the effect of predator stress in the right ACo. In contrast, CPP increased the predator stress enhancement of ACo pCREB-lir in the left hemisphere (Table 3.1b, Figure 3.6). In the PCo, pCREB levels were reduced by predator stress in both hemispheres. In the right hemisphere, CPP had no effect on this reduction but in the left

hemisphere, CPP partially returned pCREB-lir values to control levels (Table 3.1b, Figure 3.6).

Mean Contrasts were also done within nuclei across hemispheres. Except in the PCo, all nuclei within the middle AP plane range showed elevated pCREB-lir in the left hemisphere compared to the right hemisphere in the ECPP group [all Bonferroni protected $t(10)>5.45$, $p<0.05$; Figures 3.4, 3.5, 3.6]. In addition to the ECPP group, both the VC group (Bonferroni protected $t>2.44$, $p<0.05$) and EC group (Bonferroni protected $t>2.67$, $p<0.05$) showed elevated pCREB-lir in the left hemisphere compared to the right hemisphere in the BLv (Figure 3.4). The PCo nucleus showed a different hemispheric pCREB-lir pattern (Figure 3.6). Specifically, in the EC group, right hemisphere pCREB-lir exceeded left hemisphere pCREB-lir [Bonferroni protected $t(22)=2.72$, $p<0.05$]. Conversely, in the VC group, left hemisphere pCREB-lir exceeded right hemisphere pCREB-lir [Bonferroni protected $t(10)=2.33$, $p<0.05$].

3.4.2.3 Posterior AP Plane Range (greater than 2.68 mm posterior to bregma)

The cat exposed (E) and cat exposed plus vehicle (VE) differed in this AP plane range so they could not be combined. There was a three-way Group x Nucleus x Side interaction [$F(21,112)=2.62$, $p<0.001$]. To determine the nature of the interaction, mean contrasts were made among the four groups (VC, E, VE, ECPP) for each nucleus and in each hemisphere. Planned comparisons were done using t-tests and other comparisons were completed with Bonferroni protected t-tests. The t and p values for each comparison can be found in Table 3.1c.

Across groups, there was a common pattern of pCREB-lir in the BLa, BLv, BM, La and PCo in both hemispheres (Figure 3.7). Unlike most patterns seen in the anterior and middle ranges, predator stress only (E) suppressed pCREB-lir relative to handled vehicle controls (VC), except in the BM and BLv where they did not differ. Vehicle plus predator stress reversed the predator stress-induced suppression, returning pCREB-lir to control levels in La or elevating it above control levels in the remaining nuclei. CPP plus predator stress (ECPP) further elevated the vehicle plus predator stress (VE) increase in pCREB-lir in all nuclei in both hemispheres, with the exception of the BLv, where ECPP and VE pCREB-lir were equally elevated above VC and E (Table 3.1c, Figure 3.7).

The Ce showed a somewhat similar pCREB pattern across groups. In both hemispheres, predator stress reduced pCREB-lir as compared to all other groups, which did not differ (Table 3.1c, Figure 3.8). Thus, vehicle plus predator stress (VE) blocked the predator stress-induced reduction in pCREB-lir and CPP acted like vehicle indicating an injection, but no significant drug, effect.

Similar to the pattern of pCREB-lir in the nuclei discussed above, predator stress also reduced pCREB-lir in the left and right ACo and the left Me (Table 3.1c, Figure 3.9). As in other nuclei, vehicle plus predator stress (VE) reversed the predator stress-induced effects on pCREB-lir, increasing pCREB-lir above control in all loci except the left ACo. CPP reduced the vehicle increase of pCREB-lir in the right ACo and Me, had no effect in left ACo, and potentiated the vehicle injection effect in the left Me (Table 3.1c, Figure 3.9).

Comparing nuclei across hemispheres, there were no hemispheric differences in any groups in the Ce, BLv, BM, and BLa (all $t<1.76$, $p>0.05$). In the La and PCo, both the VE and ECPP groups showed greater pCREB-lir in the left hemisphere (all $t(10)>2.04$, $p<0.05$; Figure 3.7). In the ACo, the VC group showed greater pCREB-lir in the left hemisphere (Bonferroni protected $t=3.42$, $p<0.05$; Figure 3.9). Finally, in the Me, the VE group showed greater pCREB-lir in the right hemisphere [Bonferroni protected $t(10)=4.79$, $p<0.05$; Figure 3.9].

3.4.3 PAG Densitometry pCREB Analysis

To assess the effects of predator stress and NMDA receptor block on pCREB-lir in the dorsal and ventral PAG, relative OD unit data were analyzed with a three-way mixed ANOVA assessing Group (E, VE, VC and ECPP), Hemisphere, and AP plane with repeated measures on Hemisphere and AP plane. The PAG was divided into two AP planes, an anterior AP plane range and a posterior AP plane range. In addition, each column was analyzed separately. There was an AP plane effect for the dorsal and ventral columns of the PAG [all $F(1,21)>7.77$, $p<0.05$]. The posterior AP range expressed more pCREB-lir than the anterior range in all groups and in both columns (Figure 3.10).

Right and left hemispheres were analyzed separately for the lPAG because Adamec et al. (2003) showed a right/left difference in pCREB-lir after predator stress. In the left hemisphere, there was an AP plane effect [$F(1,21)=30.16$, $p<0.001$] but no Group, or Group interactions [all $F(3,21)<1.29$, $p>0.05$; Figure 3.10].

Since pCREB-lir is enhanced after predator stress in the right lPAG (Adamec et al., 2003), t-tests were done to compare predator stressed groups and controls. Both AP planes had the same pattern of pCREB-lir in the right lateral column so they were combined for simplicity. The VC and ECPP group did not differ [t(22)=0.664, p>0.05] nor did the VE and E groups [t(22)=1.0111, p>0.1]. However, ECPP and VC groups differed significantly from both E and VE animals [t(22) = 2.29, $p < .02$; Figure 3.11]. Consistent with the previous study (Adamec et al., 2003), predator stress increased pCREB-lir in the right lPAG compared to controls. Importantly, CPP blocked the increase in predator stress-induced enhancement of pCREB-lir.

3.4.4 BNST pCREB Analysis

The BNST was divided into two AP ranges, an anterior range (0.26 mm to 0.40 mm posterior to bregma) and a posterior region (0.92 mm posterior to bregma). For the anterior range, the BNST was divided into three nuclei: lateral, medial and ventral (as described in Paxinos & Watson, 1982). A separate analysis was done for the two AP plane ranges.

For the anterior BNST range, relative OD unit data were analyzed with a three-way mixed ANOVA assessing Group, Hemisphere and BNST Nucleus with repeated measures on Hemisphere and Nucleus. There was a two-way Group x Nucleus interaction [F(6,38)=3.05, p<0.05]. E and VE groups showed decreased pCREB-lir compared to ECPP or VC groups in all three nuclei (Tukey-Kramer, p<0.05; Figure 3.12 top panel). While nuclei did not differ in the exposed groups (E and VE), VC and ECPP

groups displayed the greatest pCREB-lir in the lateral nucleus, lowest in the ventral and intermediate in the medial BNST.

For the posterior BNST, relative OD unit data were analyzed with a two-way ANOVA assessing Group and Hemisphere. There was a main Group effect [$F(3,18)=166.37$, $p<0.001$]. Similar to the anterior BNST, pCREB-lir was suppressed equally in E or VE groups and CPP returned pCREB-lir to control levels. pCREB-lir in the VC and ECPP groups were equal in a given nucleus, suggesting that the reduction in pCREB-lir in BNST is NMDA receptor-dependent (Tukey-Kramer, $p<0.05$; Figure 3.12 bottom panel).

3.4.5 DMH and ACC pCREB Analysis

There were no main effects or interactions of pCREB-lir in the DMH or ACC from two-way ANOVAs assessing Group by Hemisphere with repeated measures on Hemisphere (data not shown).

3.4.6 PAG Right Lateral Column pCREB-lir Stereology Analysis

This analysis was done to examine if the densitometry differences described above in the right lPAG reflected differences in numbers of cells stained for pCREB. Since Adamec et al. (2003) found no differences in cell numbers in the right lPAG but found a difference in pCREB density, we developed a modified stereological cell counting technique to count pCREB-lir stained cells. To do this, we divided the cells into

two categories; dark/black stained cells and all other stained cells, to see if numbers of densely stained cells might account for the densitometry differences.

Tissue volumes were adjusted for shrinkage, and had a mean volume of 0.54 mm^3. The non-parametric Kruskal-Wallis test was used in all cases involving non-normal data sets (omnibus normality of residuals > 14.17, $p < 0.001$). Volumes did not differ statistically (Kruskal-Wallis one-way ANOVA on Ranks H(3)=1.74, p>.05). There were also no differences in tissue thickness across groups ($F = 0.55$, $p > 0.6$).

3.4.6.1 Dark (Black) Stained Cells

Stereoinvestigator provided an estimate of the total number of cells stained darkly (black) for pCREB. Dark/black cells were observed in only two of the 24 rats. Interestingly, both rats showing dark/black cells were in the cat exposed only group (E), and no dark/black cells were counted in the remaining 3 groups (VE, ECPP, VC). Exposed animals (E) had a mean density of 2147 cells/mm^3. This did not differ statistically from the other three groups (Kruskal-Wallis one-way ANOVA on Ranks H(3)=6.26, p>.09, corrected for ties). The overall mean ± S.E.M. Schaeffer CE value was 0.022 ± 0.01, which suggests a high level of confidence in predicting that population values were estimated without bias.

3.4.6.2 All Remaining Stained Cells

Stereoinvestigator was used to obtain an estimate of the total number of cells stained for pCREB (other than black/dark cells) in the right lPAG. Density of cells was

calculated as described above. Treatment groups (VE, ECPP, VC, E) did not differ statistically from one another in the density of lighter stained cells ($H(3)=1.04$, $p>0.78$). Schaeffer CE values did not differ between groups (.035 ± .001), which suggests that population values were estimated without bias.

3.4.6.3 Combined Cell Counting

Since there was no difference in numbers of dark stained cells and lighter stained cells across groups, we combined all cells stained for pCREB (lighter + dark/black cells). The stereoinvestigator provided an estimate of the total number of cells stained for pCREB in the right lPAG.

Stereoinvestigator estimates of total cells stained for pCREB were divided by the adjusted volume estimate to produce a cell density measure of cells/mm^3. Kruskal-Wallis One Way ANOVA on Ranks indicated that cell densities did not differ statistically across groups ($H(3)= 0.06$, $p>.09$). This result is consistent with previous research in which differences in pCREB density were found but no differences were found in the numbers of cells stained for pCREB in the amygdala and PAG (Adamec et al., 2003; Adamec et al., *in press*).

3.5 Discussion

A five minute unprotected exposure to a cat increases ALB in a rat that lasts for at least three weeks (Adamec & Shallow, 1993; Adamec et al., 2001; Blundell et al., 2005 - Chapter 2), or longer (Cohen, Zohar, Matar, Zeev, Loewenthal, & Richter-Levin, 2004).

Moreover, most anxiety-like behavioral changes following predator stress are NMDA receptor-dependent (Adamec et al., 1999a; Adamec et al., 1999b; Blundell et al., 2005 - Chapter 2). Since phosphorylation of CREB is regulated by NMDA receptors (Segal & Murphy, 1998) and pCREB-lir is increased after predator stress (Adamec et al., 2001; Adamec et al., 2003; Adamec et al., *in press*), we examined the effects of CPP on predator stress-induced changes in pCREB-lir in several brain areas. Results show that CPP blocked the predator stress-induced increase in pCREB-lir in the right lPAG; blocked, enhanced or had no effect on the predator stress-induced changes in pCREB-lir in all amygdala nuclei; reversed the predator stress-induced suppression of pCREB-lir in the BNST; and had no effect on pCREB-lir in the DMH or ACC. Importantly, changes in pCREB-lir produced by predator stress (or CPP) in all amygdala nuclei and the PAG were hemisphere- and AP plane-dependent.

3.5.1 NMDA Receptor-Dependent pCREB Changes in the Brain

3.5.1.1 NMDA Receptor-Dependent pCREB Changes in the Amygdala - Relationship to Lasting Anxiogenic Effects of Predator Stress

A variety of research has found changes in pCREB/CREB-lir in the amygdala after fear induction (Bilang-Bleuel et al., 2002; Davies et al., 2004; Hall et al., 2001; Pandey, Roy, & Zhang, 2003; Shen et al., 2004; Stanciu et al., 2001). This is consistent with previous findings of increased pCREB-lir in the mid AP plane range of several amygdala nuclei including the Ce, BLa, BM, and La after predator stress (Adamec et al., *in press*).

As mentioned above, most predator stress-induced lasting increases in ALB can be blocked with NMDA receptor antagonists given systemically or microinfused directly into the amygdala (Adamec et al., 1999a; Adamec et al., 1999b; Blundell et al., 2005 - Chapter 2). If pCREB-mediated processes in the amygdala underlie NMDA receptor-dependent effects of predator stress on behavior, one would expect predator stress to change amygdala pCREB-lir in an NMDA receptor-dependent manner. Results confirm this hypothesis in several amygdala nuclei including the anterior Ce, BAOT, Me, ACo, right BLa, and right La (Figure 3.2 & 3.3). In addition, the NMDA receptor-dependent increase in pCREB-lir after predator stress was also displayed in the mid AP plane range of the right BM, right BLa, and right BLv (Figure 3.4). This is consistent with the work of Wallace, Stellitano, Neve, and Duman (2004) which showed that over-expression of CREB in mid BLa increased ALB as measured in the EPM. It is important to note, however, that we assessed pCREB levels, *not* alterations in CREB. This is a significant distinction as changes in pCREB-lir may occur independently of changes in total CREB levels, as observed by Bilang-Bleuel et al. (2002) after swim stress.

Since most anxiogenic effects of predator stress are NMDA-receptor dependent, the current findings support the hypothesis that pCREB-lir changes in the amygdala may mediate lasting neural changes underlying most long-lasting anxiogenic effects of predator stress. Importantly, this conclusion applies to specific amygdala nuclei within a particular AP plane and hemisphere. Further discussion of AP plane and hemispheric differences in pCREB-lir appears below.

3.5.1.2 NMDA Receptor-Dependent pCREB Changes in the PAG - Relationship to Lasting Anxiogenic Effects of Predator Stress

The PAG has been implicated in rodent ALB (Brandao, Anseloni, Pandossio, de Araujo & Castilho, 1999) and is activated by predator stress (Canteras & Gotto, 1999). Moreover, Adamec et al. (2003) have shown increased pCREB-lir in the right lPAG following predator stress. Since NMDA receptor antagonists are anxiolytic when microinjected into the PAG (Molchanv & Guimaraes, 2002) and phosphorylation of CREB is regulated by NMDA receptors (Segal & Murphy, 1998), we examined the effects of CPP on predator stress-induced increases in pCREB-lir in the PAG. CPP blocked the predator stress-induced increase in pCREB-lir in the right lPAG (Figure 3.11) suggesting that phosphorylation of CREB in the right lPAG may contribute to the lasting increases in ALB after predator stress. Importantly, both predator stress and CPP had no effect on pCREB-lir in any other column of the PAG. Implications of changes in pCREB-lir in the right lPAG will be discussed below.

3.5.1.3 NMDA Receptor-Dependent pCREB Changes in the BNST - Relationship to Lasting Anxiogenic Effects of Predator Stress

Previous research has implicated the BNST in fear-related behaviors. For instance, Walker and Davis (1997) blocked glutamate receptors within the BNST and observed a disruption of light-enhanced startle. Because bright light is an unlearned aversive stimulus, they suggest that the BNST is involved in unconditioned fear. Consistent with this notion, temporary inactivation of the BNST blocked 2,5-dihydro-

2,4,5-trimethylthiazoline (TMT - a component of fox feces)-induced freezing (Fendt et al., 2003). Furthermore, studies have shown c-*fos* elevation in several brain areas, including the BNST, following exposure to predator odors (Day, Masini, & Campeau, 2004; Dielenberg, Hunt, & McGregor, 2001b; Figueiredo, Bodie, Tauchi, Dolgas, & Herman, 2003). Thus, we measured pCREB-lir in the BNST after predator stress. Since NMDA receptors are in the BNST (Gracy & Pickel, 1995) and light-enhanced startle can be blocked with an NMDA antagonist infused into the BNST (Walker and Davis, 1997), we wished to determine whether changes in pCREB-lir after predator stress in the BNST were NMDA receptor-dependent. As was seen in several amygdala nuclei and the right lPAG, predator stress-induced changes in pCREB-lir were reversed with CPP (Figure 3.12). Since anxiogenic effects of predator stress are NMDA receptor-dependent and pCREB changes in the BNST are reversed with CPP, this suggests that the BNST may indeed be involved in ALB produced by predator stress. Unlike *c-fos* results, however, predator stress in the present study suppressed pCREB-lir in the BNST relative to controls and CPP rats. This is consistent with reduced pCREB levels in the BNST of animals that display an elevated fear potentiated startle response (Meloni, Jackson, Cohen, & Carlezon, 2003). Yet, it is difficult to determine the role that the BNST plays in the anxiogenic response to predator stress and exactly what a suppression in pCREB-lir implies. Thus, further studies including increasing CREB levels by viral vectoring CREB into the BNST and/or blocking pCREB via protein kinase inhibitors in the BNST are necessary to determine the role it plays in the ALB.

3.5.2 pCREB and NMDA Receptor-Dependent LTP-like Changes After Predator Stress

In addition to behavioral changes, predator stress produces potentiation in neural transmission from the Ce to the lPAG and from VAB to the BLa (Adamec, et al., 2001; Adamec et al., 2003; Adamec, et al., 2005a). More importantly, this enhanced potentiation is blocked by NMDA receptor antagonists (Adamec et al., 2005b). Furthermore, amygdala afferent and efferent LTP-like changes are highly predictive of severity of change in ALB following predator stress (Adamec et al., 2003; Adamec et al., 2005a) and LTP-like changes in these pathways have been proposed as a mechanism mediating stress-induced changes in ALB.

3.5.2.1 pCREB and NMDA Receptor-Dependent LTP-like Changes in Ce-lPAG Pathway

Several lines of research suggest that pCREB mediates LTP-like changes in Ce-lPAG transmission, particularly the right Ce-lPAG pathway. First, pCREB-lir has been associated with long-lasting potentiation of neural transmission (Silva et al., 1998). Second, predator stress increases pCREB-lir in the right lPAG (Adamec et al., 2003). Third, predator stress-induced LTP-like changes in the Ce-lPAG are longer-lived in the right hemisphere (Adamec et al., 2003). Fourth, the same aspects of the stressor experience and reaction to it are predictive of both degree of pCREB-lir in the right lPAG and degree of potentiation in the right Ce-lPAG pathway (Adamec et al., 2003). Results from the present study replicate previous findings that predator stress increases pCREB-lir in the right lPAG (Adamec et al., 2003). If pCREB-mediated processes underlie LTP-

like changes in the Ce-lPAG pathway, one might expect predator stress to increase pCREB-lir in this pathway in an NMDA receptor-dependent manner. Indeed, as can be seen in Figure 3.11, predator stress-induced increases in pCREB-lir in the right lPAG are blocked by CPP. Like predator stress-induced increases in pCREB-lir, the longer-lived LTP-like change in Ce-lPAG is also NMDA receptor-dependent and thus, present findings provide further support for the idea that long-lasting right Ce-lPAG LTP-like changes are pCREB-mediated.

NMDA receptor-dependent predator stress-induced increases were not observed in mid-posterior Ce (the site of stimulation in previous LTP studies, Adamec et al., 2001; Adamec et al., 2003; Adamec et al., 2005a; Adamec et al., 2005b). In fact, predator stress suppressed pCREB-lir in mid Ce in the right hemisphere (Figure 3.8). Interestingly, Pandey et al. (2003) showed that in certain circumstances, pCREB suppression via microinjection of a PKA inhibitor into mid-posterior Ce, produced increased ALB as measured in the EPM. In the present study, the suppression of pCREB in the mid-posterior Ce does not appear to be NMDA receptor-dependent. These results suggest that phosphorylation of CREB is most likely involved in the predator stress-induced Ce-lPAG potentiation within the lPAG, and not the Ce, a finding consistent with other evidence that Ce-lPAG LTP-like changes are mediated post-synaptically (Adamec et al., 2001).

3.5.2.2 pCREB and NMDA Receptor-Dependent LTP-like Changes in VAB-BLa Pathway

If pCREB-mediated processes underlie LTP-like changes in the VAB-BLa pathway, one might expect predator stress to increase pCREB-lir in posterior BLa where the potentiation was recorded (Adamec et al., 2005a). The increases in pCREB-lir should also be NMDA receptor-dependent, as are predator stress-induced VAB-BLa LTP-like changes in the right hemisphere (Adamec et al., 2005b). Yet, predator stress suppressed pCREB-lir in posterior BLa in both hemispheres in the AP plane corresponding to the recording sites in the BLa. Therefore, pCREB changes in amygdala cells are likely not involved in VAB-BLa potentiation. This conclusion is consistent with Maren and Fanselow's (1995) finding that NMDA receptor-dependent LTP in the VAB-BLa pathway may be presynaptic. Moreover, this result is somewhat consistent with results in Adamec et al. (2001) that showed a trend in paired pulse evidence for pre-synaptic changes in VAB-BLa transmission nine days after predator stress.

3.5.3 Non-NMDA Receptor-Dependent Predator Stress Changes in pCREB

As discussed above, predator stress-induced changes in pCREB-lir in several amygdala nuclei are NMDA receptor-dependent, as are most anxiety-like behavioral changes following predator stress (Adamec et al., 1999a; Adamec et al., 1999b; Blundell et al., 2005 - Chapter 2). Therefore, these particular brain areas may mediate lasting neural changes underlying most of the long-lasting ALB produced by predator stress. However, results from other amygdala nuclei in the anterior, mid and posterior AP plane ranges are not consistent with this hypothesis. For example, although pCREB-lir is

elevated by predator stress in the mid ACo, left BM, left BLa and left BLv (Figures 3.5, 3.6), changes in pCREB-lir do not appear NMDA receptor-dependent. Amygdala areas demonstrating this pattern may be implicated in behavioral changes produced by predator stress which are not NMDA receptor-dependent. For example, increases in ALB as measured in the social interaction test which are not NMDA receptor-dependent may be mediated by changes in NMDA receptor-independent pCREB (Blundell et al., 2005 - Chapter 2). Furthermore, pCREB-lir in anterior left BLa and La, and the mid Ce, La, and Me did not differ from controls, suggesting that pCREB-lir in those amygdala nuclei (hemisphere and AP plane specific) in the present conditions likely do not mediate the anxiety-like behavioral changes.

Findings in the Ce and La are inconsistent with Adamec et al. (*in press*) who reported predator stress-induced increases in pCREB-lir in the Ce and La (in the corresponding AP plane range). There are several possible reasons for this discrepancy. First, and most obvious, predator stress effects on pCREB-lir in the Ce, La and Me are not reliable in these nuclei. Second, there were minor methodological differences between this study and the previous study. Yet, these differences are unlikely to be the cause as other nuclei (BLa, BM and ACo) display a similar pattern of pCREB-lir following predator stress in both studies. Finally, different patterns of anxiogenic effects have been found after predator stress (Adamec et al., 2001; Blundell et al., 2005 - Chapter 2). For instance, Blundell et al. (2005) used the same predator stress model in the same laboratory and unlike Adamec et al. (2001), failed to find increased peak startle amplitude after predator stress. This is consistent with the idea that different brain areas

may be modified by predator stress to control different behavioral changes. Adamec et al. (1999b) provide evidence for this view by showing that the effects of pre-stress local amygdala NMDA receptor block on ALB depends on both the type of behavior measured and the hemisphere of injection. Unfortunately, it is difficult to test this hypothesis using pCREB staining as animals are sacrificed soon after predator stress in order to determine pCREB levels and thus, their behavioral profiles cannot be determined. However, studies examining the behavioral effects of pCREB/CREB manipulation, via over-expression of CREB in specific brain areas, are warranted to help understand the influence of changes in pCREB within a specific brain area on ALB.

In addition to changes in the anterior and mid amygdala, changes in pCREB-lir were also seen in the posterior amygdala. Specifically, pCREB-lir in the predator stress alone and predator stress plus vehicle groups differed, unlike in the anterior and mid AP plane ranges. Curiously, predator stress alone suppressed pCREB-lir in most amygdala nuclei (i.e., PCo, BLa, La, Ce, and ACo); in pther nuclei pCREB-lir did not differ from controls (BM, BLv and Me). Yet, the vehicle plus predator stress reversed the predator stress suppression of pCREB bringing pCREB-lir to control levels or above control levels (Figures 3.7, 3.8, 3.9). Since previous studies consistently find no differences in ALB between vehicle plus predator stress and predator stress alone (Blundell et al., 2005 - Chapter 2; Adamec et al., 2005b), this may suggest that phosphorylation of CREB in the posterior AP plane range of the amygdala likely does not mediate lasting neural changes underlying most long-lasting increases in ALB produced by predator stress.

3.5.4 Hemispheric and AP Plane Differences in pCREB

To our knowledge, this is the first study to show both hemispheric and AP plane differences in pCREB-lir in the amygdala after stress. Adamec et al. (*in press*) have previously found no hemispheric asymmetries in predator stress-induced pCREB-lir in amygdala nuclei. It is important to note, however, that predator stress-induced pCREB-lir did not differ across hemisphere in the corresponding mid AP plane range in the present study. The only differences in pCREB-lir between hemispheres were found in the PCo and BLv, two nuclei not examined in the Adamec et al. (*in press*) study. Hemispheric differences in pCREB-lir after predator stress are not unique to the amygdala, rather, they have also been found in the PAG (Adamec et al., 2003; present study). In addition to alterations in pCREB-lir, behavioral effects of limbic sensitization produced by kindling also show hemispheric asymmetries. Adamec and colleagues have demonstrated that kindling left BLa is anxiolytic, whereas kindling right BLa is anxiogenic (Adamec, Blundell, & Burton, 2004; Adamec & Morgan, 1994). Importantly, hemispheric differences in the amygdala have also been found in human PTSD patients. For example, Vietnam veterans suffering from PTSD show increased PET activation in the right amygdala in response to trauma-related stimuli (Shin et al., 1997). This is consistent with Rauch and colleagues who found increased blood flow in right-sided limbic, paralimbic and visual areas following traumatic reminders (Rauch et al., 1996).

In addition to hemispheric asymmetries, differences were also seen in pCREB-lir in amygdala nuclei across the three AP plane ranges. For example, the Ce, Me, ACo,

BLa, and La all showed increased pCREB-lir in response to predator stress in the anterior AP plane compared to controls. In the posterior AP plane range, however, all nuclei show suppressed pCREB-lir following predator stress compared to controls. These results are particularly important as most studies only examine pCREB-lir in a nucleus in one AP plane. The variability in pCREB-lir across AP plane in amygdala nuclei may be understood in the context of the circuitry in which the cells are embedded. It may be inappropriate to treat any anatomically defined nucleus of the amygdala as a functional unit when examining pCREB-lir (Adamec et al., 2004). For example, circuitry in which mid areas of the BLa are embedded may differ from those of more anterior or posterior planes of the BLa (McDonald, 1996; McDonald, Mascagni, & Guo, 1996; Savander, Go, LeDoux, & Pitkänen, 1995). One might expect a variable amount of pCREB-lir depending on the circuitry involved. In our laboratory, this is not the first study to show differential responses of amygdala nuclei depending on AP plane. Adamec et al. (2004) have shown that amygdala nuclei, depending on AP plane, produce different behavioral effects when kindled. Similar to the amygdala, AP plane differences in CREB have also been found in the VTA. In an elegant study, Olson and colleagues found that chronic exposure to drugs of abuse induces CREB activity throughout the VTA (Olson, Zabetian, Bolanos, Edwards, Barrot, Eisch, Hughes, Self, Neve, & Nestler, 2005). Importantly, they showed that CREB activation within the rostral versus caudal sub-regions of the VTA produced opposite effects on drug reward. In particular, increased CREB in rostral portions of the VTA increases the rewarding effects of cocaine and morphine, whereas similar changes in the caudal portion have the opposite effects. An important next step in

understanding the AP plane-dependent alterations in pCREB-lir in the present study is to increase or decrease pCREB in particular amygdala nuclei in a specific AP plane position to assess its effect on ALB.

Another unusual finding which requires further study is the elevation in pCREB-lir in the mid left BM, BLa, Ce, La, Me, ACo, and posterior BM, PCo, BLa, BLv, La, and left Me in predator stressed rats given CPP compared to predator stressed rats alone (see Figures 3.4-3.9). It is unclear what this increase implies and thus, further studies involving the effects of local injection of CPP into these brain areas on behavior are necessary to help clarify these results.

4.5.5 Densitometry Versus Stereological Cell Counting

Previous studies have shown that predator stress increases the density of pCREB staining in individual cells but does not increase the number of cells stained for pCREB in the amygdala (Adamec et al., *in press*) and lPAG (Adamec et al., 2003). These results are consistent with Swank (2000) who examined pCREB-lir in mouse cortex and amygdala after taste aversion learning and found changes in density of pCREB staining unaccompanied by increases in numbers of cells stained for pCREB. To better understand these results, we assessed pCREB staining in the right lPAG using a modified stereological procedure in which darkly stained cells were counted separately from all other stained cells. Increased numbers of dark (black) cells could provide an explanation for the reduced light transmission observed in the right lPAG after predator stress in the present study and past studies (Adamec et al., 2003, Adamec et al., *in press*). Yet, this

was not found, as total number of lighter and dark (black) cells did not differ across groups, indicating that although pCREB staining is more dense in the right lPAG of predator stressed rats, the lack of a difference in cell numbers across groups cannot be explained by increased numbers of darkly stained cells. This suggests that intermediate stained cells promote the densitometry differences and that intensity of moderately stained cells is higher in predator stressed rats than control or CPP rats. When all pCREB stained cells were combined and counted (dark cells plus all other stained cells), there were still no group differences, consistent with previous studies (Adamec et al., 2003; Adamec et al., *in press*). Because of this, we suggest that cell counting alone is not sufficient for detecting changes in phosphorylation of CREB in all cases.

3.5.6 Conclusions

It is clear in the amygdala, PAG, and BNST that the pattern of pCREB-lir in the present experimental conditions are hemisphere- and AP plane-dependent. Furthermore, the BLa, BM, Ce, lPAG, and BNST areas where predator stress changes pCREB-lir in an NMDA receptor-dependent manner are candidate areas of neuroplastic change contributing to lasting changes in ALB. In addition, pCREB changes in the amygdala appear to be unrelated to NMDA receptor-dependent predator stress-induced LTP-like changes in amygdala afferent (VAB-BLa) and efferent (Ce-lPAG) pathways. In contrast, pCREB changes in the right lPAG appear closely linked to NMDA receptor-dependent predator stress-induced LTP-like changes in the right Ce-lPAG pathway. Finally, consistent with previous results from the amygdala and PAG (Adamec et al., 2003;

Adamec et al., *in press*), predator stress increases the amount of pCREB-lir within each PAG cell, but does not increase the number of PAG cells stained for pCREB

Figure Captions

Figure 3.1. Top left, photomicrograph of pCREB expression in a section of the PAG in the Predator Stressed group. Top right, photomicrograph of a section of the PAG from the Predator Stressed group that was stained without the primary antibody. There is some ventricular swelling in the control section due to the freezing process. Bottom left, photomicrograph of pCREB expression in a section of the amygdala in the Predator Stressed group. Bottom right, photomicrograph of a section of the amygdala in the Predator Stressed group where the primary antibody was saturated with pCREB prior to staining

Figure 3.2. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units - Relative Optical Density) in BAOT, Ce, Me, and ACo nuclei between groups {vehicle handled (VC), combined stress [vehicle plus predator stress (VE) and predator stress only (E)], predator stress plus CPP (ECPP)} are graphed in AP plane range = 1.8 mm posterior to bregma. Means marked with the same letter do not differ but differ from those with different letters in within each hemisphere. Means marked with a # differ from the corresponding group in the other hemisphere. Me – medial nucleus; ACo – anterior cortical nucleus; Ce – central nucleus, BAOT - bed nucleus of the accessory olfactory tract.

Figure 3.3. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units - Relative Optical Density) in BLa and La nuclei between groups {vehicle handled (VC), combined stress [vehicle plus predator stress (VE) and predator stress only (EC)], predator stress plus CPP (ECPP)} are graphed in AP plane range = 1.8 mm posterior to bregma. Means marked with the same letter do not differ but differ from those with different letters within each hemisphere. Means marked with a # differ from the corresponding group in the other hemisphere. BLa – basolateral nucleus; La– lateral amygdala

Figure 3.4. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units - Relative Optical Density) in BM, BLa, BLv nuclei between groups {vehicle handled (VC), combined stress [vehicle plus predator stress (VE) and predator stress only (E)], predator stress plus CPP (ECPP)} are graphed in AP plane range 2.12 to 2.68 mm posterior to bregma. Means marked with the same letter do not differ but differ from those with different letters within each hemisphere. Means marked with a * differ from the corresponding control with a 1-tailed t-test. Means marked with a # differ from the corresponding group in the other hemisphere. BM – basomedial nucleus; BLa – basolateral nucleus; BLv - basolateral ventral.

Figure 3.5. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units - Relative Optical Density) in Ce, La, and Me nuclei between groups {vehicle handled (VC), combined stress [vehicle plus predator stress (VE) and predator stress only (E)], predator stress plus CPP (ECPP)} are graphed in AP plane range 2.12 to 2.68 mm posterior to bregma. Means marked with the same letter do not differ but differ from those with different letters within each hemisphere. Means marked with a # differ from the corresponding group in the other hemisphere. Ce - central amygdala, La - lateral amygdala, Me - medial amygdala.

Figure 3.6. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units - Relative Optical Density) in ACo and PCo nuclei between groups {vehicle handled (VC), combined stress [vehicle plus predator stress (VE) and predator stress only (E)], predator stress plus CPP(ECPP)} are graphed in AP plane range 2.12 to 2.68 mm posterior to bregma. Means marked with the same letter do not differ but differ from those with different letters within each hemisphere. Means marked with a # differ from the corresponding group in the other hemisphere. ACo – anterior cortical; PCo - posterior cortical.

Figure 3.7. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units - Relative Optical Density) in BM, PCo, BLa, La and BLv nuclei between groups [vehicle handled (VC), predator stress only (E), vehicle plus predator stress (VE), predator stress plus CPP (ECPP)] are graphed in AP plane range > 2.68 mm posterior to bregma. Means marked with the same letter do not differ but differ from those with different letters within each hemisphere. Means marked with a # differ from the corresponding group in the other hemisphere. BM - basomedial; PCo - posterior cortical; BLa - basolateral; BLv - basolateral ventral; La - lateral.

Figure 3.8. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units - Relative Optical Density) in Ce nucleus between groups [vehicle handled (VC), predator stress only (E), vehicle plus predator stress (VE), and predator stress plus CPP (ECPP)] are graphed in AP plane range > 2.68 mm posterior to bregma. Means marked with the same letter do not differ but differ from those with different letters within each hemisphere. Ce- central amygdala.

Figure 3.9. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units - Relative Optical Density) in ACo and Me nuclei between groups [vehicle handled (VC), predator stress only (E), vehicle plus predator stress (VE), predator stress plus CPP (ECPP)] are graphed in AP plane range > 2.68 mm posterior to bregma. Means marked with the same letter do not differ but differ from those with different letters within each hemisphere. Means marked with a # differ from the corresponding group in the other hemisphere. ACo - anterior cortical; Me- medial.

Figure 3.10. Mean + SEM relative optical density units (PAG optical density units divided by total section optical density units - Relative Optical Density) in all three columns (Dorsal, Ventral and left Lateral) in both AP planes (AP1 and AP2) are graphed. Means marked with the same letter do not differ but differ from those with different letters within the same column (Tukey-Kramer tests, $p<0.05$).

Figure 3.11. Mean + SEM relative optical density units (PAG optical density units divided by total section optical density units - Relative Optical Density) in the right lateral column for each group [vehicle control (VC), predator stress only (E) , vehicle predator stress (VE), CPP plus predator stress(ECPP)] are graphed. Comparisons were made within the same column between groups. Means marked with the same letter do not differ but differ from those with different letters.

Figure 3.12. In the top panel, Mean + SEM relative optical density units (BNST optical density units divided by internal capsule optical density units - Relative Optical Density) in BNST nuclei(Medial, Lateral, Ventral BNST) between groups (vehicle control (VC), predator stress only (E), vehicle plus predator stress (VE), CPP plus predator stress(ECPP)] collapsed over hemisphere and side are graphed. Means marked with the same letter do not differ but differ from those with different letters (Tukey-Kramer, $p<0.05$). In the bottom panel, Mean + SEM relative optical density units (BNST optical density units divided by full section optical density units - Relative Optical Density) in posterior BNST between groups [vehicle control (VC), predator stress only (E), vehicle plus predator stress (VE), CPP plus predator stress (ECPP)] collapsed over hemisphere and side are graphed. Means marked with the same letter do not differ but differ from those with different letters (Tukey-Kramer, $p<0.05$).

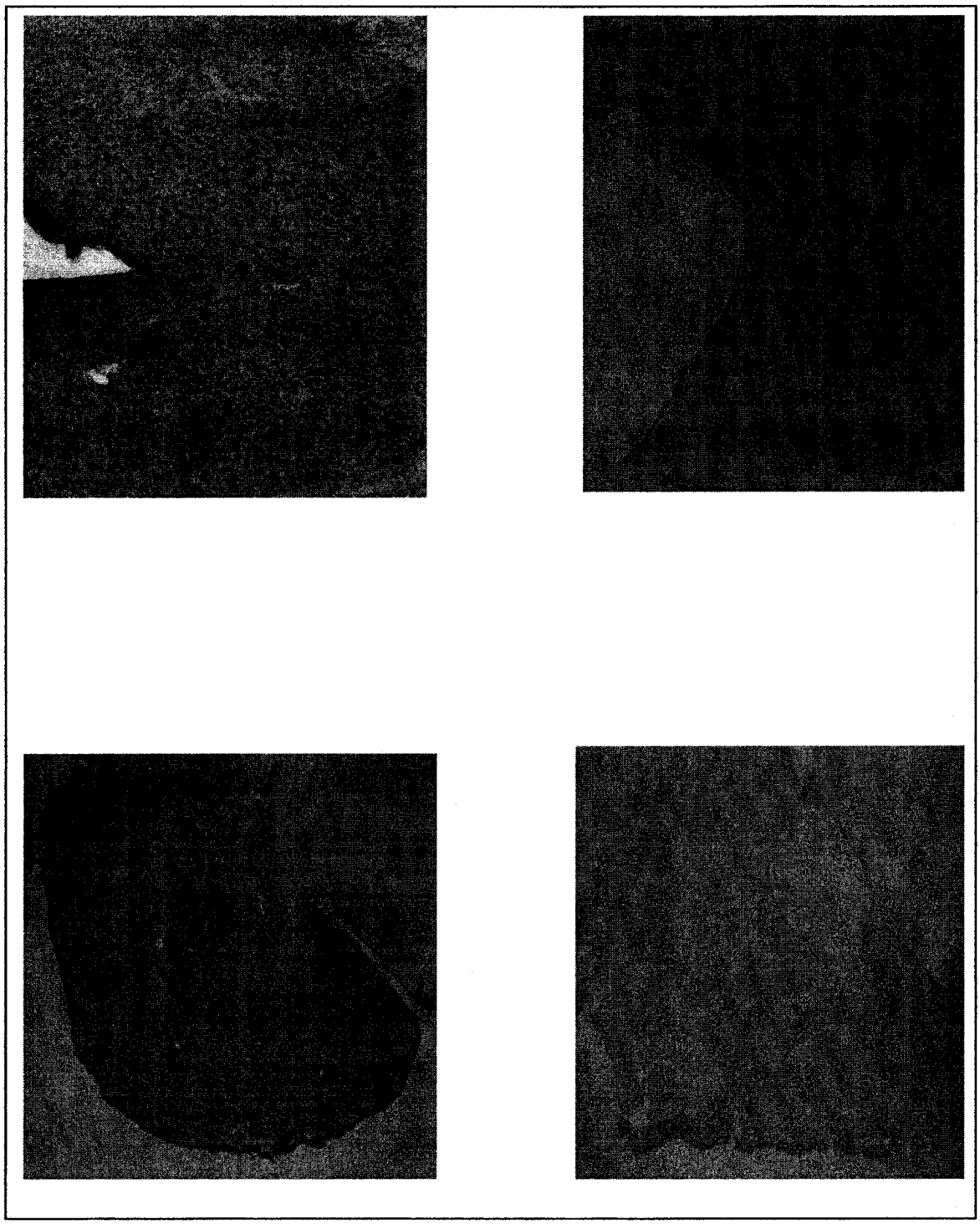


Figure 3.1

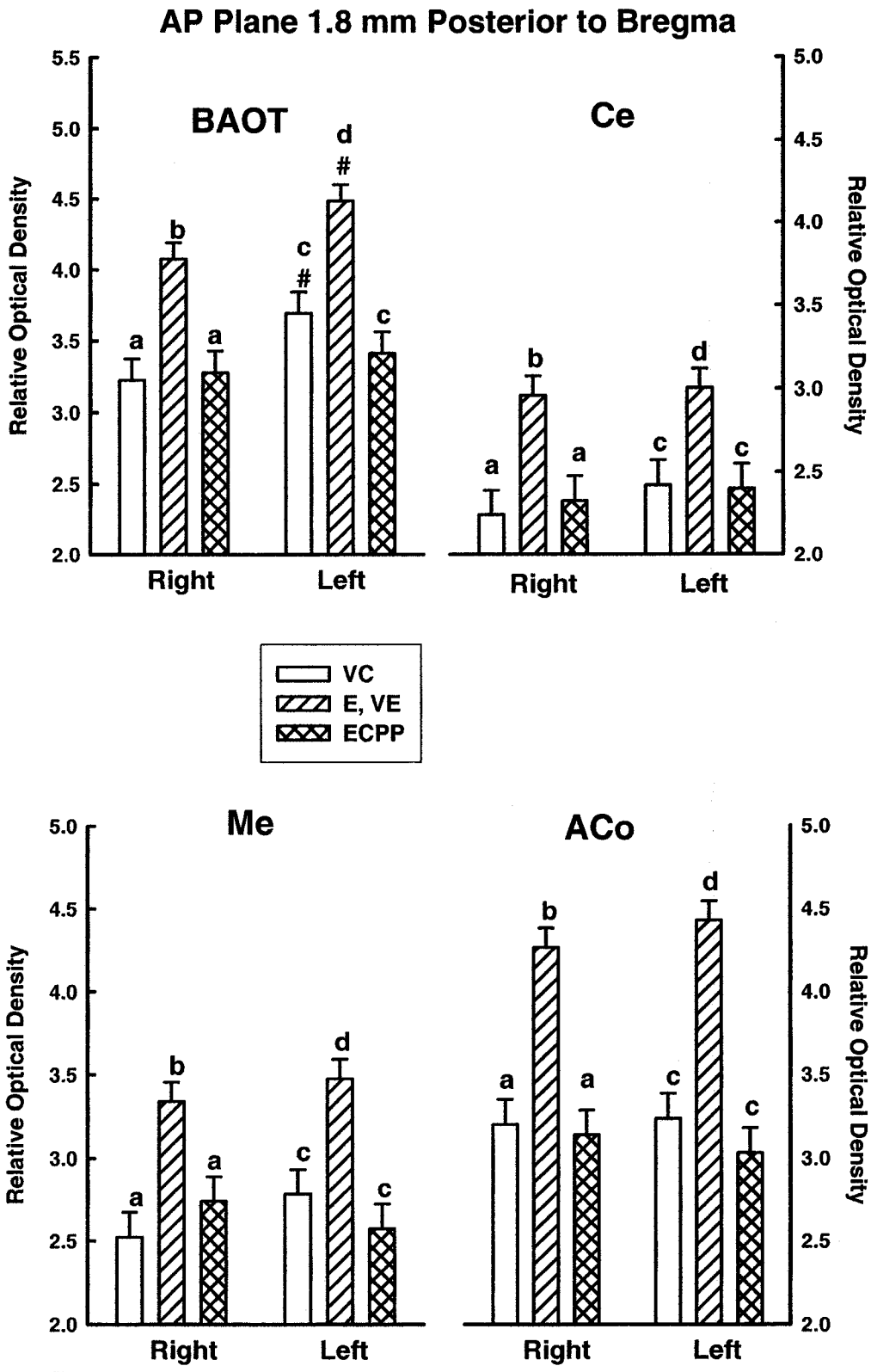

AP Plane 1.8 mm Posterior to Bregma
BAOT
Ce
Me
ACo
Relative Optical Density
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
a
b
a
c
#
d
#
c
Right
Left
VC
E, VE
ECPP

Figure 3.2

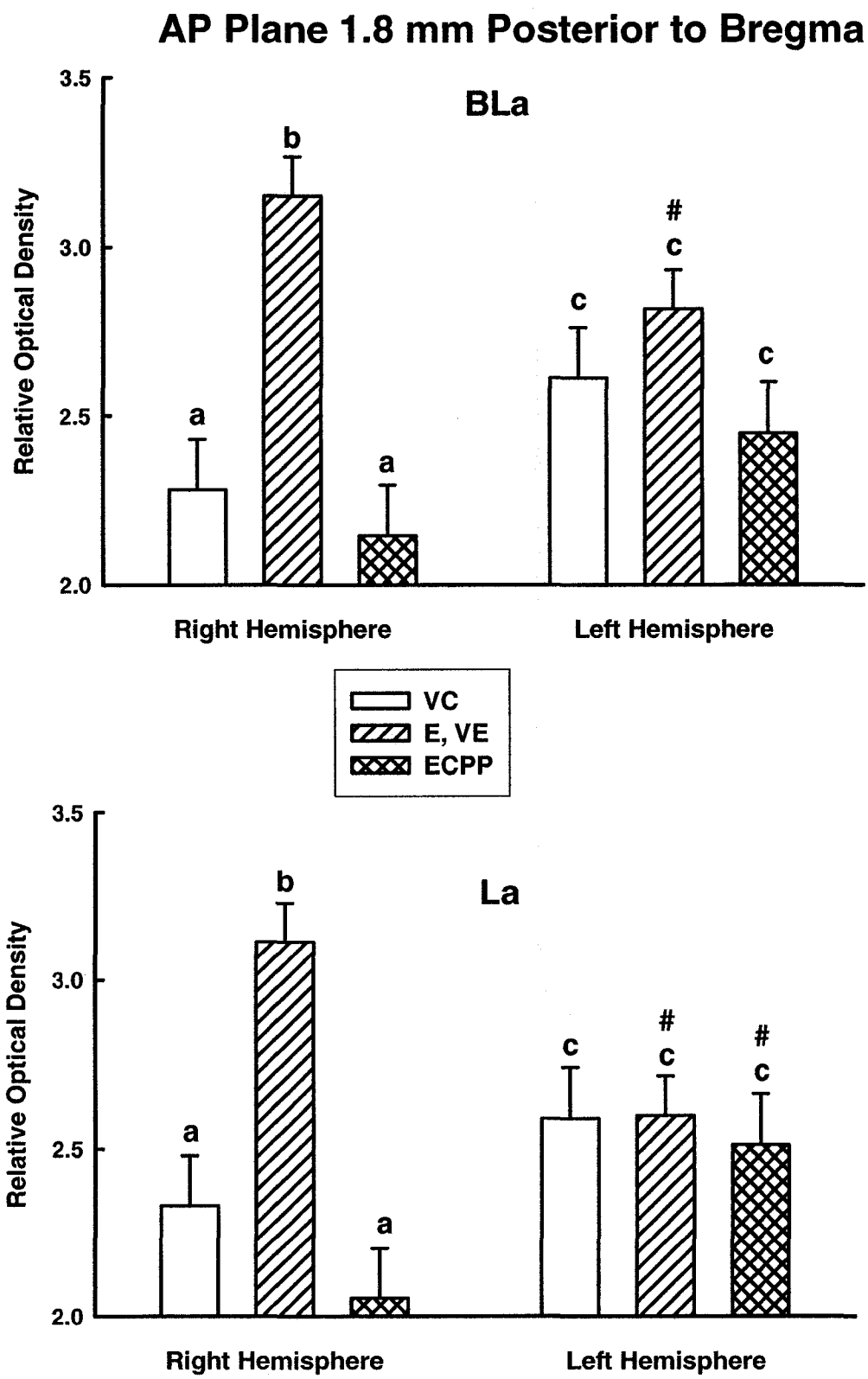

AP Plane 1.8 mm Posterior to Bregma
BLa
Relative Optical Density
3.5
3.0
2.5
2.0
a
b
a
c
#
c
c
Right Hemisphere
Left Hemisphere
VC
E, VE
ECPP
La
Relative Optical Density
3.5
3.0
2.5
2.0
a
b
a
c
#
c
#
c
Right Hemisphere
Left Hemisphere

Figure 3.3

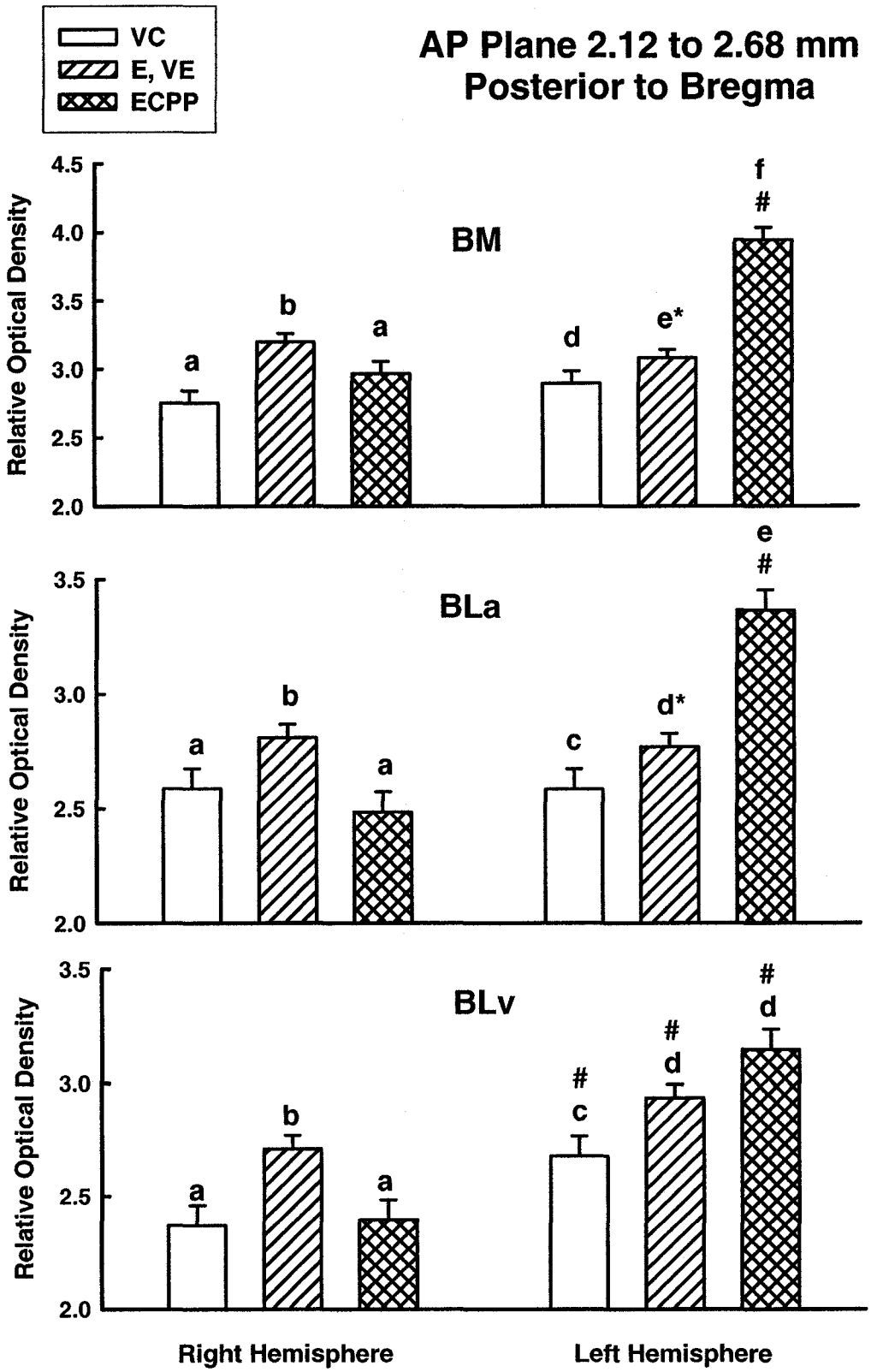

VC
E, VE
ECPP
AP Plane 2.12 to 2.68 mm
Posterior to Bregma
BM
BLa
BLv
Relative Optical Density
4.5
4.0
3.5
3.0
2.5
2.0
a
b
a
d
e*
f
#
BM
a
b
a
c
d*
e
#
BLa
a
b
a
#
c
#
d
#
d
BLv
Right Hemisphere
Left Hemisphere

Figure 3.4

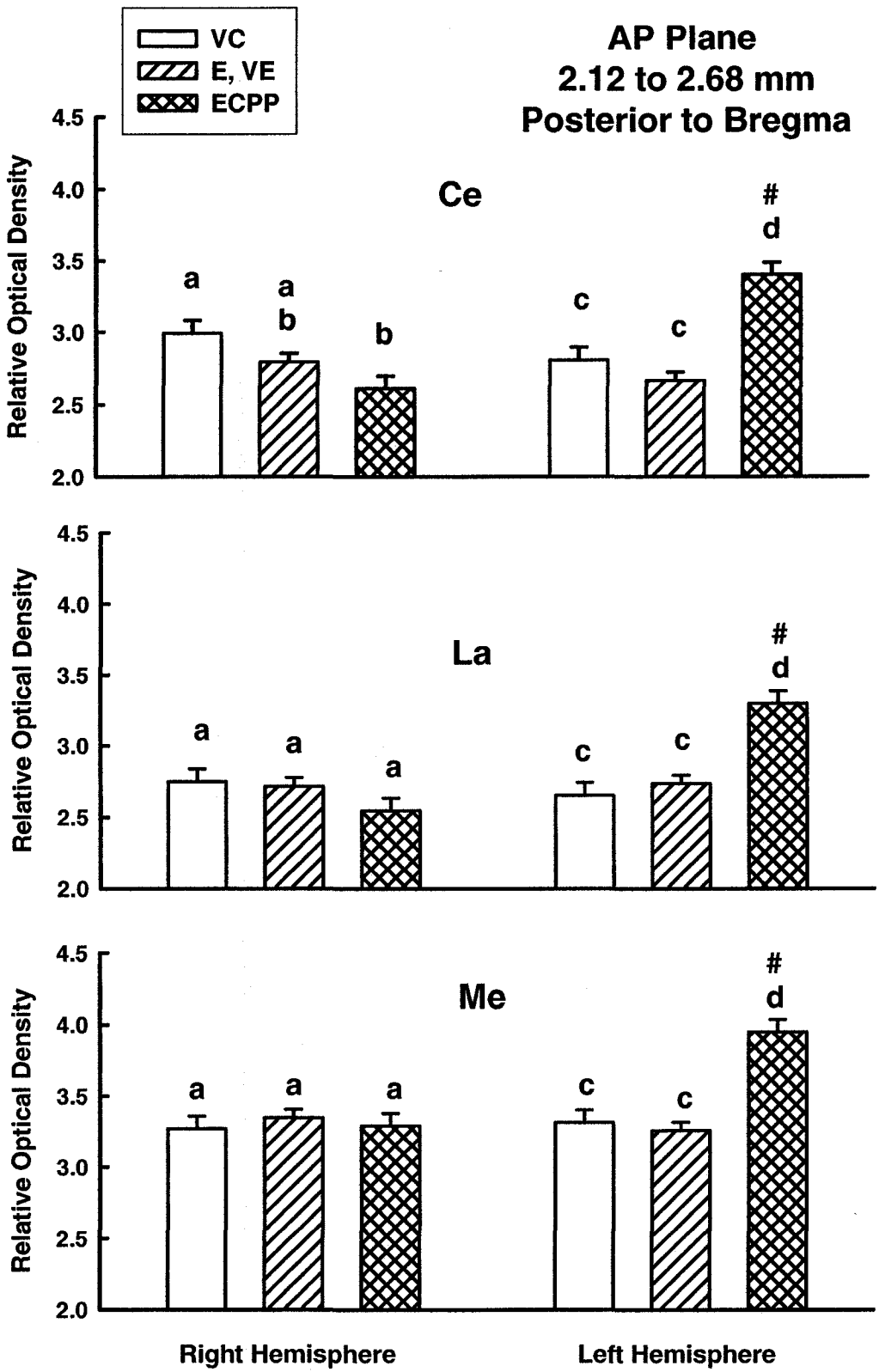

VC
E, VE
ECPP
AP Plane
2.12 to 2.68 mm
Posterior to Bregma
Ce
La
Me
Relative Optical Density
4.5
4.0
3.5
3.0
2.5
2.0
a
a
b
b
c
c
#
d
Right Hemisphere
Left Hemisphere

Figure 3.5

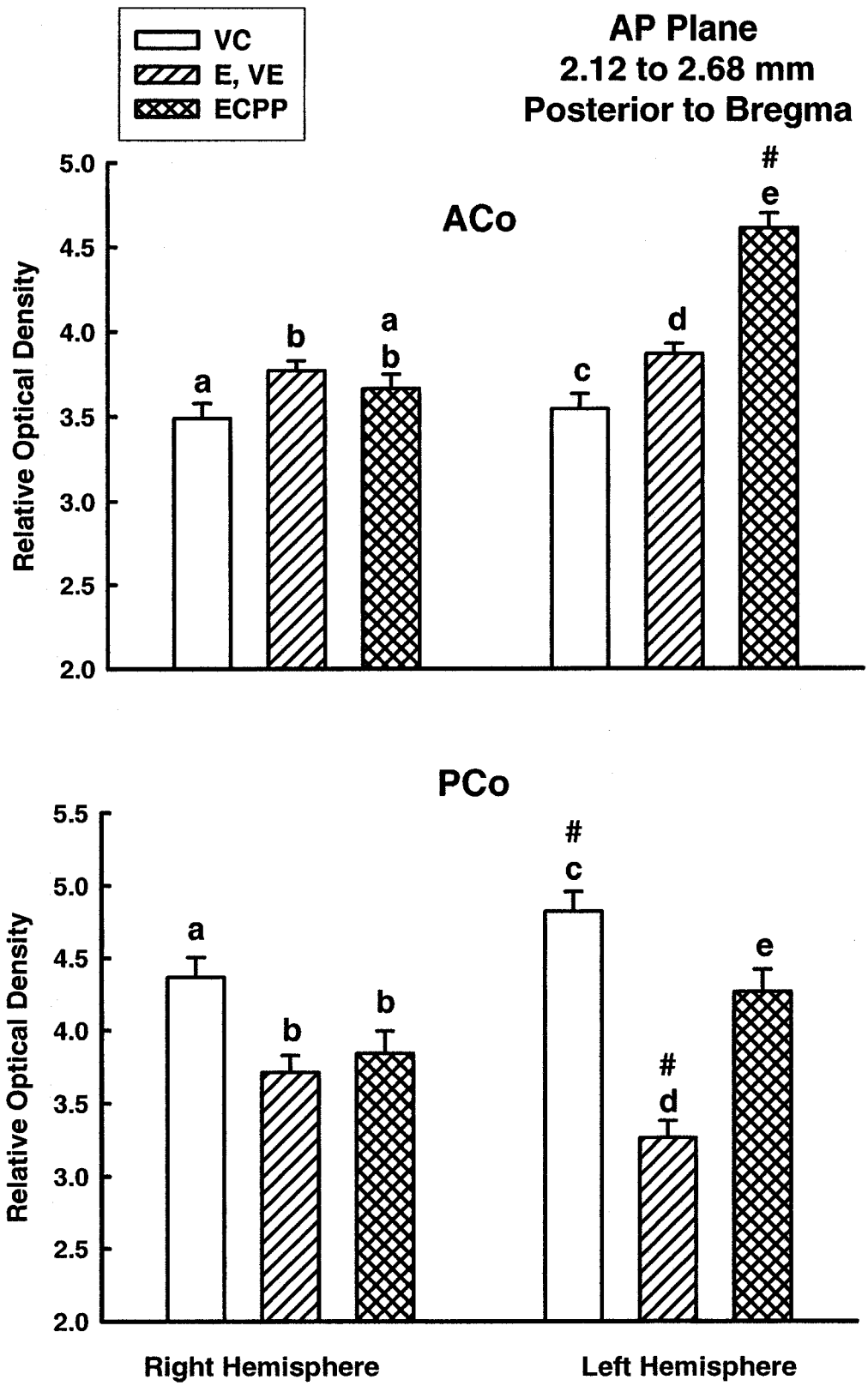

VC
E, VE
ECPP
AP Plane
2.12 to 2.68 mm
Posterior to Bregma
ACo
Relative Optical Density
5.0
4.5
4.0
3.5
3.0
2.5
2.0
a
b
a
b
c
d
#
e
PCo
Relative Optical Density
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
a
b
b
#
c
#
d
e
Right Hemisphere
Left Hemisphere

Figure 3.6

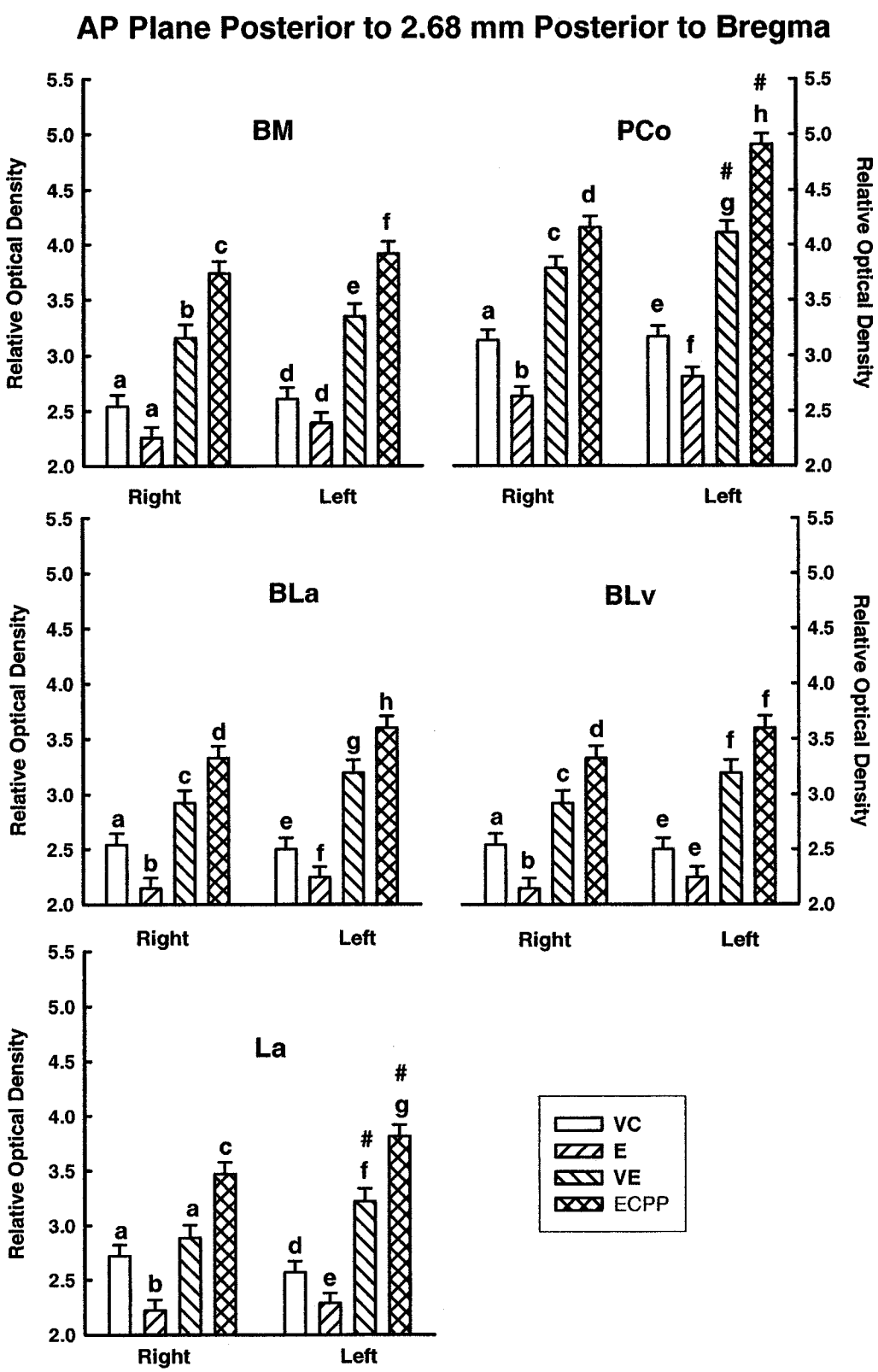

AP Plane Posterior to 2.68 mm Posterior to Bregma
BM
PCo
BLa
BLv
La
Relative Optical Density
Right
Left
VC
E
VE
ECPP

Figure 3.7

AP Plane Posterior to 2.68 mm Posterior to Bregma

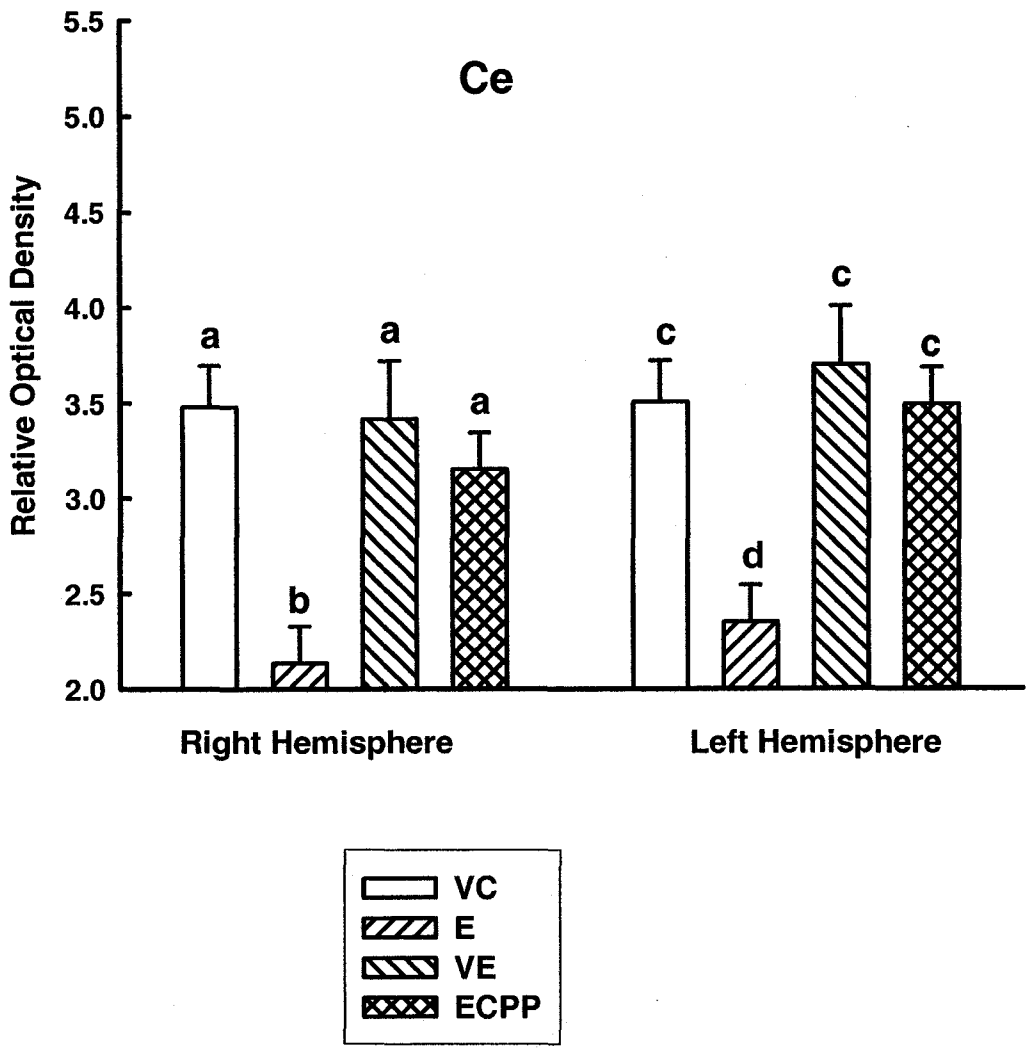


Figure 3.8

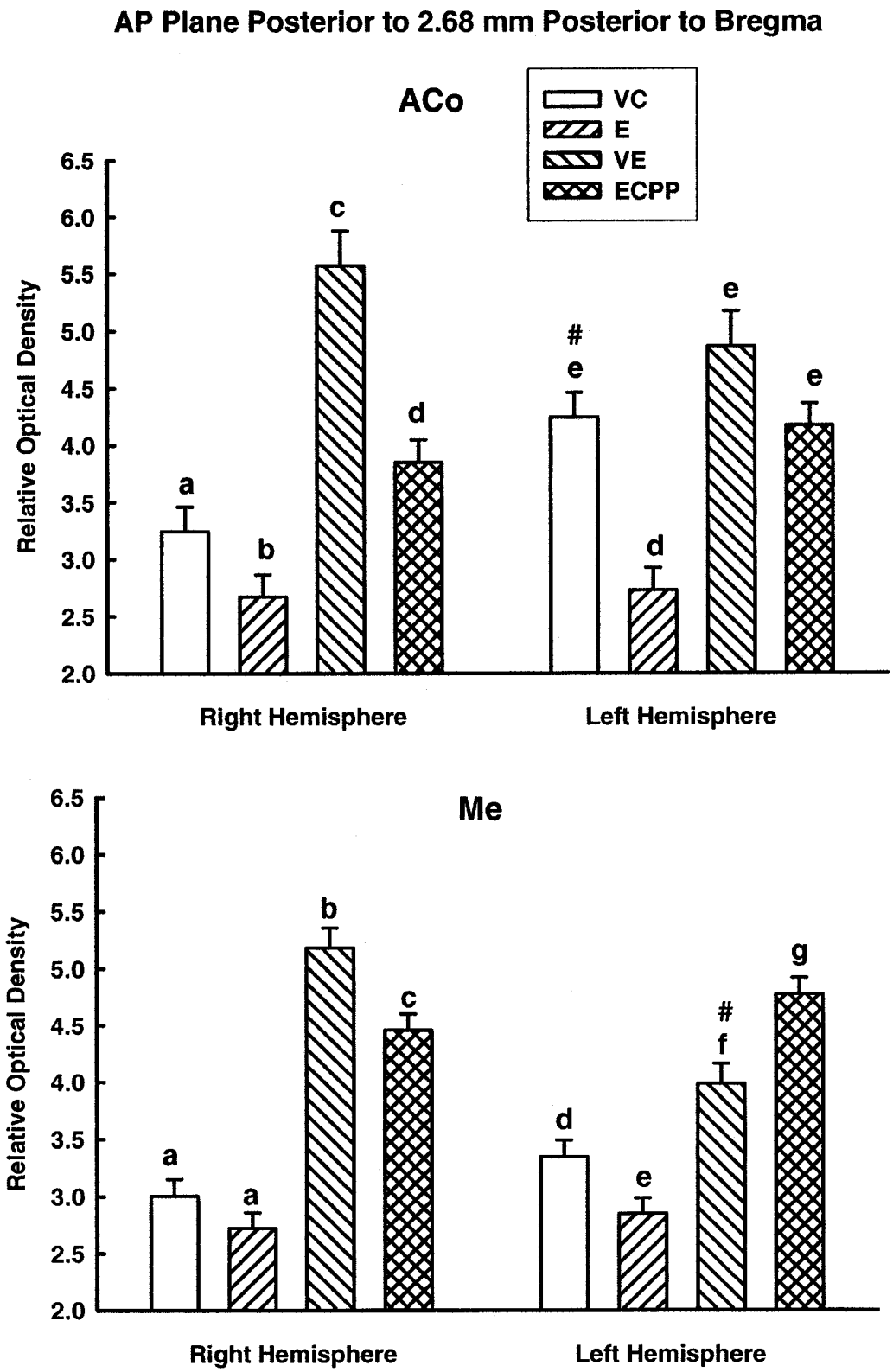

AP Plane Posterior to 2.68 mm Posterior to Bregma
ACo
VC
E
VE
ECPP
Relative Optical Density
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
a
b
c
d
#
e
d
e
e
Right Hemisphere
Left Hemisphere
Me
Relative Optical Density
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
a
a
b
c
d
e
#
f
g
Right Hemisphere
Left Hemisphere

Figure 3.9

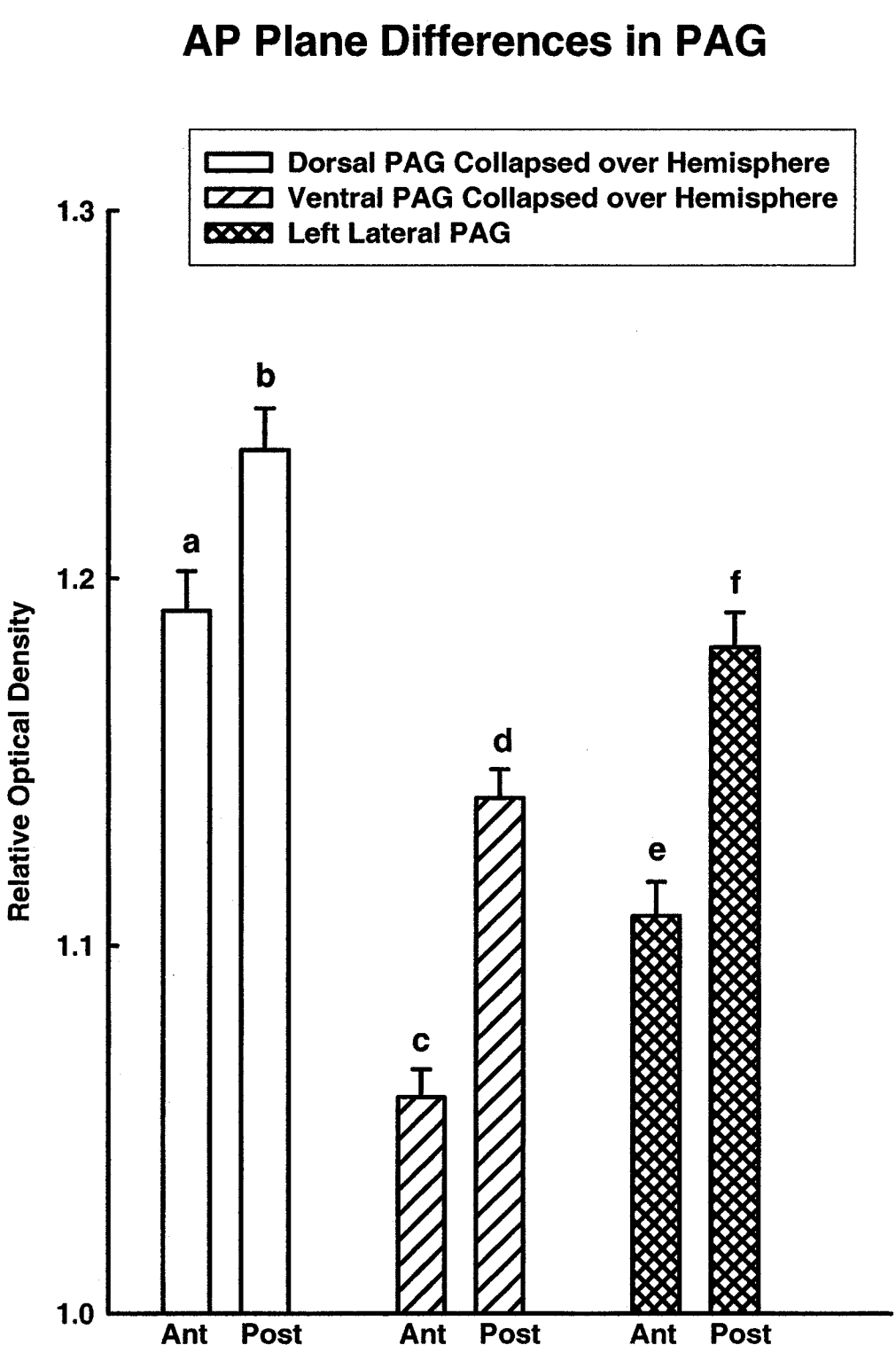

AP Plane Differences in PAG
Dorsal PAG Collapsed over Hemisphere
Ventral PAG Collapsed over Hemisphere
Left Lateral PAG
Relative Optical Density
1.3
1.2
1.1
1.0
a
b
c
d
e
f
Ant
Post
Ant
Post
Ant
Post

Figure 3.10

Right Lateral PAG over all AP Planes

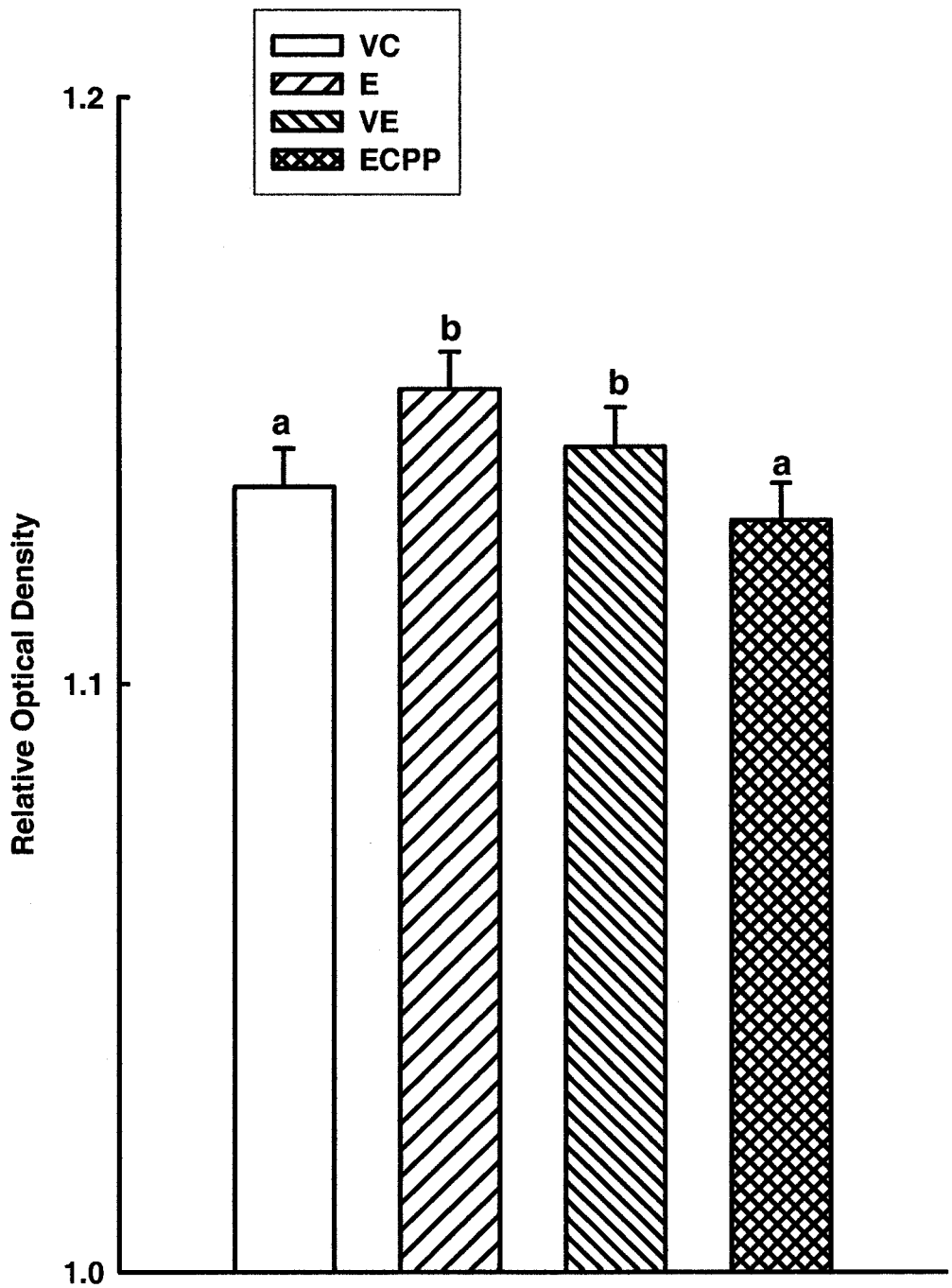
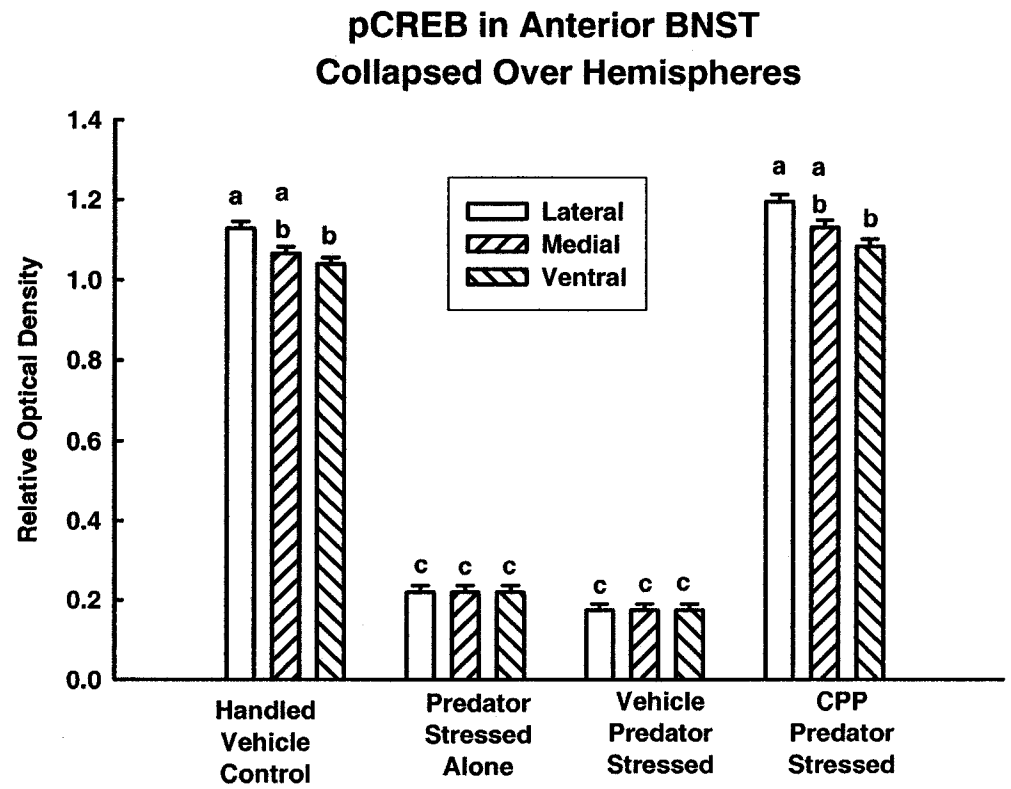


Figure 3.11


pCREB in Anterior BNST
Collapsed Over Hemispheres
Relative Optical Density
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Lateral
Medial
Ventral
a a b b
c c c
c c c
a a b b
Handled Vehicle Control
Predator Stressed Alone
Vehicle Predator Stressed
CPP Predator Stressed

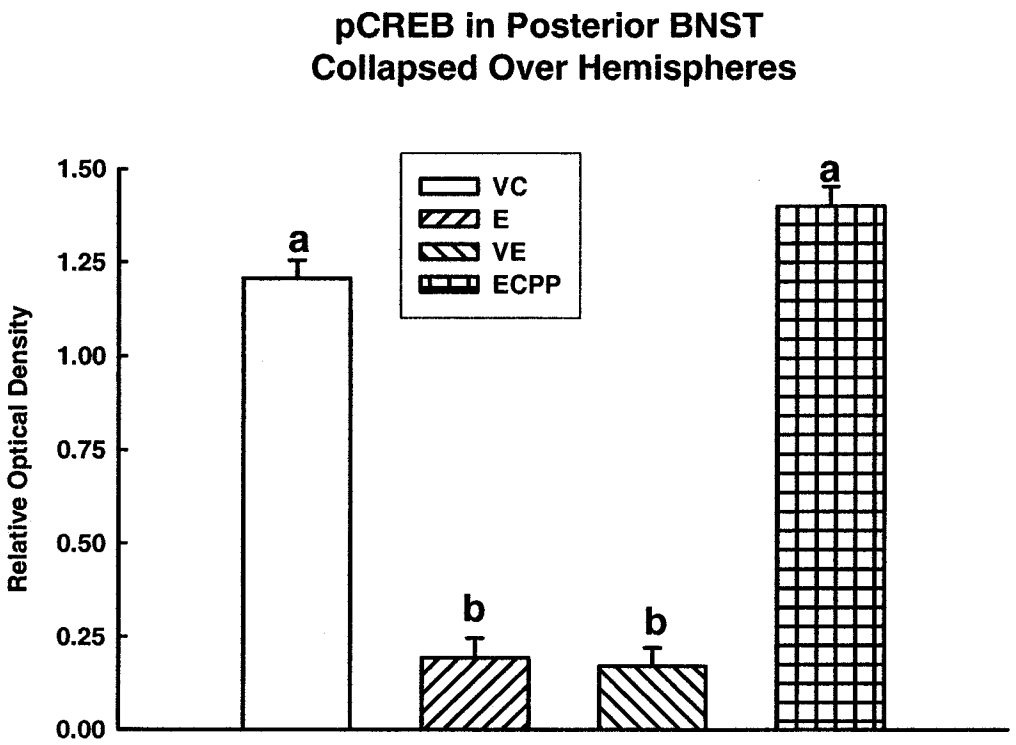

pCREB in Posterior BNST
Collapsed Over Hemispheres
Relative Optical Density
1.50
1.25
1.00
0.75
0.50
0.25
0.00
VC
E
VE
ECPP
a
b
b
a

Figure 3.12

Table 3.1a. Anterior AP Plane

Side	Nuclei	Comparisons						Figure
		VC v E		VC v ECPP		ECPP v E		
		t value	p value	t value	p value	t value	p value	
Right	Ce	3.8	≤0.05*	0.5	ns	3.35	≤0.05*	3.2
Right	ACo	5.63	≤0.05*	0.3	ns	5.96	≤0.05*	3.2
Right	Me	4.32	≤0.05*	1.02	ns	3.18	≤0.05*	3.2
Right	BAOT	4.49	≤0.05*	0.24	ns	4.21	≤0.05*	3.2
Right	La	4.14	≤0.05*	1.31	ns	5.6	≤0.05*	3.3
Right	BLa	4.6	≤0.05*	0.64	ns	5.31	≤0.05*	3.3
Left	Ce	3.08	≤0.05*	0.11	ns	3.2	≤0.05*	3.2
Left	ACo	6.3	≤0.05*	0.97	ns	7.39	≤0.05*	3.2
Left	Me	3.66	≤0.05*	0.99	ns	4.77	≤0.05*	3.2
Left	BAOT	4.18	≤0.05*	1.33	ns	5.67	≤0.05*	3.2
Left	La	0.05	ns	0.36	ns	0.46	≤0.05*	3.3
Left	BLa	1.08	ns	0.77	ns	1.94	ns	3.3

Table 3.1b. Middle AP Plane

Side	Nuclei	Comparisons						Figure
		VC v E		VC v ECPP		ECPP v E		
		t value	p value	t value	p value	t value	p value	
Right	BLa	2.09	≤0.05	0.81	ns	3.04	≤0.05*	3.4
Right	BLv	3.17	≤0.05*	0.19	ns	2.95	≤0.05*	3.4
Right	BM	4.15	≤0.05*	1.69	ns	2.16	≤0.05	3.4
Right	Ce	1.88	ns	3.02	≤0.05*	1.73	ns	3.5
Right	La	0.3	ns	1.61	ns	1.59	ns	3.5
Right	Me	0.72	ns	0.17	ns	0.53	ns	3.5
Right	ACo	2.62	≤0.05*	1.42	ns	0.99	ns	3.6
Right	PCo	3.62	≤0.05*	2.53	≤0.05*	0.67	ns	3.6
Left	BLa	1.73	+	6.19	≤0.05*	5.55	≤0.05*	3.4
Left	BLv	2.4	≤0.05*	3.71	≤0.05*	1.97	ns	3.4
Left	BM	1.74	+	8.33	≤0.05*	8.06	≤0.05*	3.4
Left	Ce	1.32	ns	4.75	≤0.05*	7.09	≤0.05*	3.5
Left	La	0.77	ns	5.12	≤0.05*	5.26	≤0.05*	3.5
Left	Me	0.54	ns	5.15	≤0.05*	6.65	≤0.05*	3.5
Left	ACo	3.04	≤0.05*	8.48	≤0.05*	6.94	≤0.05*	3.6
Left	PCo	8.62	≤0.05*	2.68	≤0.05*	5.18	≤0.05*	3.6

ns= not significant
*= bonferroni protected t-test
\+ p<0.05, 1-tailed

Table 3.1c. Posterior AP Plane

Side	Nuclei	Comparisons												Figure
		VC v E		VC v VE		VC v ECPP		E v VE		ECPP v E		ECPP v VE		
		t value	p value	t value	p value	t value	p value	t value	p value	t value	p value	t value	p value	
Right	BLa	2.85	≤0.05*	2.45	≤0.05*	5.27	≤0.05*	5.17	≤0.05*	8.21	≤0.05*	2.57	≤0.05*	3.7
Right	BLv	1.32	ns	2.85	≤0.05	4.25	≤0.05*	4.16	≤0.05*	5.68	≤0.05*	1.2	ns	3.7
Right	BM	1.72	ns	4.25	≤0.05*	8.31	≤0.05*	6	≤0.05*	10.27	≤0.05*	3.66	≤0.05*	3.7
Right	Ce	4.62	≤0.05*	0.17	ns	1.13	ns	3.53	≤0.05*	3.7	≤0.05*	0.73	ns	3.8
Right	La	3.58	≤0.05*	1.08	ns	5.02	≤0.05*	4.44	≤0.05*	8.66	≤0.05*	3.67	≤0.05*	3.7
Right	Me	1.43	ns	9.51	≤0.05*	7.09	≤0.05*	10.98	≤0.05*	8.7	≤0.05*	3.71	≤0.05*	3.9
Right	ACo	1.96	≤0.05*	5.57	≤0.05*	2.08	≤0.05	7.98	≤0.05*	4.29	≤0.05*	4.74	≤0.05	3.9
Right	PCo	3.96	≤0.05*	4.63	≤0.05*	7.27	≤0.05*	8.44	≤0.05*	11.15	≤0.05*	2.48	≤0.05	3.7
Left	BLa	1.92	≤0.05	4.49	≤0.05*	7.36	≤0.05*	6.34	≤0.05*	9.39	≤0.05*	2.54	≤0.05	3.7
Left	BLv	1.91	ns	2.92	≤0.05	3.97	≤0.05*	4.79	≤0.05*	5.96	≤0.05*	0.86	ns	3.7
Left	BM	1.59	ns	4.79	≤0.05*	8.75	≤0.05*	6.43	≤0.05*	10.61	≤0.05*	3.56	≤0.05*	3.7
Left	Ce	3.97	≤0.05*	0.51	ns	0.05	ns	3.72	≤0.05*	4.16	≤0.05*	0.57	ns	3.8
Left	La	2.03	≤0.05	4.23	≤0.05*	8.35	≤0.05*	6.26	≤0.05*	10.61	≤0.05*	3.72	≤0.05*	3.7
Left	Me	2.48	≤0.05	2.79	≤0.05	6.98	≤0.05*	5.06	≤0.05*	9.64	≤0.05*	3.45	≤0.05*	3.9
Left	ACo	5.21	≤0.05*	1.65	ns	0.25	ns	5.88	<0.05*	5.26	<0.05*	1.3	ns	3.9
Left	PCo	2.88	≤0.05	6.7	≤0.05*	13.16	≤0.05*	9.61	≤0.05*	16.52	≤0.05*	5.67	≤0.05*	3.7

ns= not significant

*= bonferroni protected t-test

\+ p<0.05, 1-tailed

CHAPTER 4

Elevated pCREB in the PAG after Exposure to the Elevated Plus Maze in Rats Previously Exposed to a Cat.

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4.1 Abstract

The elevated plus maze (EPM) is an ethologically based test of anxiety-like behavior. In addition, exposure to the maze itself is stressful and anxiogenic. One of the goals of this study was to examine if the stress of EPM exposure increased pCREB-like-immunoreactivity (lir). The second goal of this study was to determine whether prior stress impacted pCREB-lir in animals exposed to the EPM. pCREB-lir was examined after exposure to the EPM in rats that had been exposed to a cat seven days earlier. Brain areas investigated for both experiments included the amygdala, periaqueductal gray (PAG), and bed nucleus of the stria terminalis (BNST), all areas considered to be part of the "fear circuit". Results show that there were no pCREB-lir differences between control rats and rats exposed to the EPM only. However, exposure to the EPM in predator stressed rats showed elevated pCREB-lir in the right lateral column of the PAG and the dorsal column of the PAG. Findings suggest mechanisms associated with neuroplasticity may be engaged by relatively mild stresses in animals with a history of severe stress exposure. This may be clinically relevant, as a key feature of posttraumatic stress disorder (PTSD) is the exaggerated reaction to a mild stressor in which the response is more appropriate to the original traumatic situation than the current conditions.

4.2 Introduction

The behavior of a rat in the elevated plus maze (EPM) is an ethologically based animal model of anxiety (Pellow, Chopin, File, & Briley, 1985). The maze consists of two closed arms and two open arms elevated approximately 50 cm from the ground. The rat is placed in the center of the maze and allowed to freely explore it (Rodgers & Cole, 1993; Rodgers & Dalvi, 1997). Several measures are taken to determine anxiety-like behavior (ALB) including open arm exploration and risk assessment (Pellow et al., 1985; Falter, Gower, & Gobert, 1992; Fernandes & File, 1996; Adamec, Blundell, & Collins, 2001). Exposure to the maze itself is stressful and anxiogenic as corticosterone levels are elevated after a five minute test (File, Zangrossi, Sanders, & Mabbutt, 1994) and re-exposure to the maze does not lead to habituation of the anxiogenic response (Pellow et al., 1985). In fact, re-exposure to the maze can produce further reductions in time spent on the open arms, perhaps indicating increased anxiety (Fernandes & File, 1996; File et al., 1998; Rodgers, Lee, & Shepard, 1992; Rodgers & Shepherd, 1993; Treit, Menard, & Royan, 1993). Moreover, corticosterone levels continue to be elevated after the second trial (File et al., 1994).

Thus, one of the goals of the present study was to examine the involvement of phosphorylated cyclic adenosine monophosphate response element binding protein (pCREB) after exposure to the EPM. Many studies have shown elevated pCREB-like-immunoreactivity (lir) in various brain areas after other stressors. For example, increases in pCREB-lir are found after predator stress (Adamec, Blundell & Burton, 2003; Adamec, Blundell & Burton, *in press*; Chapter 3), forced swimming (Bilang-Bleuel,

Rech, De Carli, Holsboer & Reul, 2002; Shen, Tsimberg, Salvadore & Meller, 2004), fear conditioning in mice (Davies, Tsui, Flannery, Li, DeLorey, & Hoffman, 2004), retrieval of a cued-fear memory (Hall, Thomas, & Everitt, 2001), and electric shock (Stanciu, Radulovic & Spiess, 2001). To our knowledge, however, pCREB-lir has not been investigated after exposure to the EPM in rats. Interestingly, Wallace and colleagues showed that in the EPM, rats over-expressing CREB in the basolateral amygdala (BLa) exhibited an increase in anxiety as measured by a significant reduction in the percent time spent in the open arms as well as a decrease in the open arm entries (Wallace, Stellitan, Neve, & Duman, 2004). Yet, Pandey and colleagues have shown that inhibiting CREB phosphorylation by infusing a protein kinase A (PKA) inhibitor into the BLa does not alter anxiety-induced states (Pandey, Roy, & Zhang, 2003). Wallace et al. (2004) suggest that this discrepancy may be a result of acute (PKA) versus chronic (viral expression) alteration of CREB function. Pandey et al. (2003) did find, however, that rats microinfused with a PKA-inhibitor into the central nucleus of amygdala (Ce) showed a significant reduction in the percentage of time spent on the open arms and in the percentage of open-arm entries compared with controls. In contrast, $CREB^{\alpha\delta-}$ deficient mice show no change in ALB as measured in the plus maze (Hebda-Bauer, Watson, & Akil, 2004). The involvement of phosphorylation of CREB in EPM anxiety is likely complex and dependent on time and neural locus-dependent. In our study, we hope to clarify the relationship between EPM anxiety and pCREB, in part, by examining pCREB-lir within several brain areas shortly after EPM exposure.

The second goal of this study was to determine whether prior stress impacts pCREB-lir following exposure to the EPM. This is particularly important as a key feature of posttraumatic stress disorder (PTSD) is the exaggerated reaction to a mild stressor in which the response is more appropriate to the original traumatic situation than the current conditions (Bremner, Krystal, Southwick, & Charney, 1995; Dykman, Ackerman, & Newton, 1997; Friedman, 1994). PTSD is a psychiatric disorder that develops following exposure to perceived severely stressful events. Recently, researchers have turned to animal models to investigate PTSD. Specifically, conditioned fear paradigms, behavior in unfamiliar situations that are fear- or anxiety-provoking and more recently, predator stress, are all models used to explore the neurobiology of psychopathologies such as PTSD (Adamec & Shallow, 1993; Adamec, Shallow, & Budgell, 1997; Cohen, Joseph & Matar, 2003; Pynoos, Ritzmann, Steinberg, Goenjian, & Prisecaru, 1996; Servatius, Ottenweller & Natelson, 1995; Stam, Bruijnzeel & Wiegant. 2000; Wang, Akirav & Richter-Levin, 2000).

In this study, we investigated changes in pCREB-lir after exposure to the EPM in naïve rats and rats previously exposed to a cat (predator stressed). Predator stress involves the unprotected exposure of a rat to a cat (Adamec & Shallow, 1993). Exposure to a cat increases ALB as measured in the EPM and this increase lasts at least three weeks (Adamec & Shallow, 1993) or longer (Cohen, Zohar, Matar, Zeev, Loewenthal, & Richter-Levin, 2004). In addition, predator stress alters pCREB-lir in the amygdala, periaqueductal gray (PAG) and bed nucleus of the stria terminalis (BNST) (Chapter 3). Furthermore, predator stressed rats show greater levels of corticosterone following

exposure to the EPM than handled controls (Adamec, Kent, Anisman, Shallow, & Merali, 1998).

Brain areas investigated after EPM exposure in naïve and predator stressed rats were areas that showed changes in pCREB-lir after predator stress (i.e., the amygdala, PAG and BNST) (Adamec et al, 2003; Adamec et al., *in press*; Chapter 3). Furthermore, a number of studies investigating the neural basis of fear-related behaviors (Fendt & Fanselow, 1999; LeDoux, 2000; Walker and Davis, 2002) have implicated brain circuitry including the amygdala, BNST, and PAG in the expression of learned and unlearned fear responses.

4.3. Methods

4.3.1 Animals

Eighteen male hooded Long Evans rats (Rattus norvegicus) from The Charles River Breeding Farms, Quebec, were used in this experiment. All rats were housed alone in clear polycarbonate cages measuring 46 cm x 24 cm x 20 cm for at least four days before testing began. Rats were given food and water *ad lib* and they were exposed to a 12-hour light/dark cycle with lights on at 7:00 AM. Rats weighed approximately 160 g on arrival and between 228 g and 275 g on the day of EPM testing. All rats were handled in the same room as their home cages for one minute a day for three days prior to testing. Handling involved picking the rat up with a gloved hand and gently holding it on the forearm. A minimal amount of pressure was used if the rat attempted to escape and the grip was released as soon as the rat became still.

4.3.2 Groups

The rats were randomly assigned to one of three groups (n=6 rats per group). The three groups were; predator stressed plus EPM (PS-EPM), handled control (HC), and handled control plus EPM (HC-EPM). Until the day of testing, all rats were treated the same. Care was taken to ensure that the rooms used to hold the rats were void of cat odor. The cat was only permitted in the exposure room.

On the day of cat exposures or handling, a multiple of three animals from each of the three groups were tested. The order of testing was counterbalanced for each set of three rats. In addition, testing began at 9:00 AM and ended at 12:00 PM with care taken to counterbalance time of treatment among the three groups. On this day, all animals were weighed immediately before testing began.

4.3.2.1 Cat Exposure (Predator Stressed)

Rats in the predator stress group (PS-EPM) were exposed to a cat. Cat exposures occurred in a large wooden room with carpet on the floor. For more details on the room, see Adamec and Shallow, (1993). The same cat was used for all rats in this experiment. The cat was placed in the room at least one hour before testing. Immediately prior to cat exposure, the rat was placed in a wooden enclosure and transported to the exposure room. The rat enclosure fits a small opening at the floor of the exposure room. A guillotine door on the enclosure was opened and the rat was gently forced to enter the room via a sliding platform inside the enclosure. The door was then closed and testing began. This method allowed the introduction of the rat into the room without handling. The five

minute exposure was videotaped to capture the activity of both the cat and the rat. Cat response to the rat ranged from watching the rat at a distance, to approach and sniffing with the occasional mild attack. Sometimes the cat pawed and bit a rat but did not physically injure it. Rats were examined for wounds after the cat encounters and none were observed.

4.3.2.2 Controls (HC and HC-EPM)

Rats in these groups did not come in contact with the cat, cat odors or rats that had previously been exposed to cats. On the day of cat testing, rats in these groups were weighed and handled for one minute. After handling, rats were placed back in their home cage and left undisturbed for one week.

4.3.3 Elevated Plus Maze Testing (EPM)

Seven days after predator stress (or handling for the control groups), ALB was tested using the hole board and EPM. The order of testing was counterbalanced between the groups (PS-EPM & HC-EPM) and testing began at 8:45 AM and ended at 11:15 AM. All tests were videotaped and measures were taken from the videotape as experimenters were hidden from view during testing. All animals were weighed immediately before testing began. The rat was first placed in the hole board for five minutes to assess activity and exploration (File & Wardill, 1975a,b). The rat was then removed and transferred by gloved hand to the EPM for a further five minutes of testing. Descriptions of the hole board and EPM can be found in Appendix 1. After testing, each rat was placed back in

its home cage and left undisturbed for 10 minutes. At this time, the rat received an intraperitoneal (ip) injection with an overdose of sodium pentobarbital (1 ml at a concentration of 65 mg/ml). Ten minutes later, the rat was checked for a reaction (if the rat still displayed a reflex, it was given a supplementary dose of 0.1 ml). When the rat displayed no reaction, it was perfused with 200 ml of heparinized saline followed by 500 ml of paraformaldehyde. The timing of the perfusion was important because it has been shown that pCREB levels peak between 20 and 25 minutes after a stimulus (Silva, Kogan, Frankland, Kida, 1998). The brain was removed, placed in a 20% sucrose solution of phosphate buffered saline (PBS) overnight and subsequently flash frozen in isopentane cooled by liquid nitrogen. The brain was left in a -70°C freezer until sectioning.

4.3.4 Handled Control (HC) Testing

On the day of testing, seven days after handling, rats in this group were weighed and handled for one minute. After handling, the rat was placed back in its home cage for ten minutes. The rat was then injected with an overdose of sodium pentobarbital and perfused as described above.

4.3.5 Behavioral Measures

4.3.5.1 Hole Board Behavioral Measures

Six behavioral measures were taken; frequency of rearing, time spent moving, number of head dips, time spent near wall, time spent in center and number of faecal boli.

Frequency of rearing and time spent in motion (time active) were measures of activity. Exploratory behavior was measured with head dips (placing the snout or head into a hole) (File & Wardill, 1975b). Time spent near the wall was a measure of thigmotaxis.

4.3.5.2 Elevated Plus Maze Behavioral Measures

Many behavioral measures were analyzed from videotape in this apparatus. Exploration and activity were scored as the number of entries into an arm of the maze (total entries) and the number of entries into the closed arms of the maze (closed arm entries). Entry occurred when the rat had all four of its feet inside one arm of the maze. Closed arm entries were further divided into closed arm returns and closed arm entry into a different closed arm.

Head dips (placing the snout or head over the side of the open arm) and rearing were also scored as independent measures of exploration. These behaviors were divided into three types; protected (rat had all four feet in closed arm for rearing or hindquarters in the closed arm for head dips), center (rat had all four feet in center of maze) and unprotected (rat had all four feet in open arm). Time spent grooming was also scored as unprotected, center, or protected.

Measures of ALB were also examined. Two measures assessed open arm exploration: ratio time and ratio entry. Ratio time was the time spent in the open arms of the maze divided by the total time spent in any arm of the maze. The smaller the ratio, the less open arm exploration and the more "anxious" the rat. Ratio entry was the number of entries into the open arms of the maze divided by the total entries into any arm

of the maze. Again, the smaller the ratio, the less the open arm exploration, the more "anxious" the rat.

Risk assessment was first defined by the Blanchards in the visible burrow system (Blanchard & Blanchard, 1989) and Adamec and Shallow (1993) were the first to adapt these measures for the EPM. Risk assessment was scored when a rat poked its head and forepaws into an open arm of the maze when its hindquarters were in a closed arm of the maze. Frequency of risk assessment was measured and converted to relative risk assessment by dividing the frequencies by the time spent in the closed arms. Fecal boli deposited in the maze were also counted.

4.3.6 Immunocytochemistry (ICC)

Forty μm frozen coronal sections were cut in a cryostat. Twelve sections were taken from 5.8 mm to 6.8 mm posterior to bregma to capture the PAG; eight sections were taken from 1.8 mm to 3.6 mm posterior to bregma to capture the amygdala. In addition, four sections were taken from 0.26 mm to 0.92 mm posterior to bregma to capture the BNST (Paxinos & Watson, 1982). These brain areas were chosen because previous studies have shown changes in pCREB-lir after predator stress (Adamec et al., 2003; Adamec et al., *in press*; Chapter 3). Anterior-Posterior (AP) plane of each section referenced to the rat brain atlas of Paxinos and Watson (1982) was determined by counting sections from the decussation of the anterior commissure (AP 0.26 mm posterior to bregma, Paxinos & Watson, 1982) to the particular section and then calculating AP plane from the section number. This permitted estimation of AP plane

position to the nearest 40 μm during cutting. A multiple of three brains, one brain from each group, was cut at the same time and brains were processed using six sections per well (sections from only one brain). All brain tissue in a run was processed in the same baths. Sections were washed with PBS, saturated with normal goat serum (NGS) and Triton X-100 in PBS, washed again, then incubated at -4°C for either 24 or 48 hrs (reused antibody) in the primary antibody (rabbit anti-rat phosphorylated CREB, 1/500 dilution). Sections were washed, then incubated in the secondary biotinylated antibody (goat anti-rabbit) followed by the avidin-biotin complex (Vector ABC kit). For visualization, diaminobenzadine was used as the chromogen (Sigma tablet). Sections were then washed, mounted onto slides, dehydrated and then cover slipped. Complete details of pCREB ICC can be found in Appendix 2.

To control for non-specific staining, the ICC procedure described above was repeated without the primary antibody (see Chapter 3, Figure 3.1, top right photomicrograph). In addition, to determine the specificity of the primary antibody, the primary antibody was saturated with pCREB prior to staining (see Chapter 3, Figure 3.1, bottom right photomicrograph). Both controls produced little or no staining.

4.3.7 Densitometry Analysis

Stained sections were analyzed blind to group assignment using image analysis software (Jandel, MOKA software). Densitometry was used to quantify the data. Hemispheres were measured separately. Examples of pCREB staining in the PAG and amygdala can be found in Chapter 3, Figure 3.1.

The PAG was divided into ventral, dorsal and lateral areas to reflect the functional columnar organization described by Bandler, Carrive, and Depaulis (1991). This was done using the aqueduct of Sylvius as a guide. Horizontal lines were drawn from the top of the aqueduct to the outside edge of the PAG and from the bottom of the aqueduct to the outside edge of the PAG for both left and right sides. The top columns were considered dorsal PAG (dPAG), the middle columns were lateral PAG (lPAG) and the bottom columns were ventral PAG (vPAG).

The amygdala was divided into its nuclei: central (Ce), basolateral (BLa), lateral (La), basomedial (BM), ventral basolateral (BLv), medial (Me), anterior cortical (ACo), posterior cortical (PCo), and bed nucleus of the accessory olfactory tract (BAOT). Nuclear boundaries were determined with templates from different AP planes defining the nuclei from the rat atlas of Paxinos and Watson, (1982). A given brain section was assigned to the nearest atlas template. Straight lined shapes (i.e., square, rectangle, triangle, rhombus, etc.) were then created from each template for all nuclei in each AP plane in order to maximize coverage of the given nucleus. These shapes were then uniformly applied to the respective nuclei in each AP plane across all groups. Coordinates set by the templates were mapped onto the actual section, which corrected for tissue shrinkage. Right and left hemispheres were measured separately using the template shapes described above.

The BNST was divided into lateral, medial and ventral within the AP plane range 0.26 mm to 0.40 mm posterior to bregma. The section that corresponded with the AP plane 0.92 mm posterior to bregma was considered posterior BNST. The same technique

described above for the amygdala was used for densitometric analysis. Again, right and left hemispheres were measured separately.

For all brain areas, raw pCREB-lir densitometry data were converted to optical densitometry (OD) units. This was done by converting the raw densitometry data to OD units via a calibrated step wedge. An image of the calibrated step wedge was taken at the same time as section images for each rat. Exponential fits of raw transmission values (x) to calibrated OD values were done by computer (Table Curve, Jandel). All fits were good (all df adjusted $r^2>.9$, $p<.01$). The exponential was then used to interpolate and convert raw transmission values to OD units. For the PAG and BNST, analyses were performed on the ratio of average OD values in particular brain areas to average OD values for the entire section. For the amygdala, analysis was performed on the ratio of average OD values of a standard one mm square sampled from the internal capsule of the hemisphere in which the amygdala measure was taken (similar to Adamec et al., *in press*).

All densitometry values from the amygdala, PAG, and BNST were analyzed separately with ANOVAs. Planned comparisons were done using t-tests and other comparisons were done using Bonferroni protected t-tests or Tukey-Kramer Multiple-Comparison Test.

4.3.8 Ethical Approval

The research methods used in this experiment were reviewed for compliance with the guidelines of the Canadian Council on Animal Care (CCAC), and approved by the Institutional Animal Care Committee of Memorial University.

4.4 Results

4.4.1 Exploration and Activity in Hole Board and EPM

One way ANOVAs revealed no Group differences in exploration (head dips) [$F(1,10)=0.05$, $p>0.05$] or activity in the hole Board (rears, time near wall, and time in center) [all $F(1,10)<4.49$, $p>0.05$]. In the EPM, however, predator stress decreased entries into the closed arm [$F(1,10)=6.76$, $p<0.05$; Figure 4.1, top panel]. This decrease in closed arm entries is likely due to increased anxiety since activity and exploration scores in the hole board did not differ between groups. File (1992) proposed that when significant effects in the number of closed arm entries were found, covariance analysis should be performed to verify if an anxiolytic effect is related to a general increase in locomotion. Thus, when ratio time and ratio entry are covaried from closed arm entries, the difference was lost [$F(1,8)=0.12$, $p>0.05$; Figure 4.1, top 'covary' panel].

4.4.2 Anxiety-Like Behavior in the EPM

There was a Group effect in open arm exploration (ratio time and ratio entry) [all $F(1,10)>5.54$, $p<0.05$]. Predator stress decreased ratio time and ratio entry into the open arms of the maze (Figure 4.1). Predator stress tended to reduce ratio risk (risk

assessment) [t(10)=1.87, p<0.10, 1-tailed; Figure 4.1 bottom panel]. In addition, predator stress reduced the ethological measure of unprotected head dips in the EPM [F(1,10)=15.96, p<0.05; Figure 4.1 bottom panel] which is consistent with past research (Adamec et al., 2001). The reduction in unprotected head dips in the predator stressed group is due to increased ALB because when ratio time and ratio entry (both measures of anxiety) are covaried, the difference is lost [F(1,8)=0.10, p>0.05].

Since exposure to the EPM has been shown to be anxiogenic (File, Zangrossi, Sanders, & Mabbutt, 1994), we wanted to determine whether this was the case in the present study in animals exposed to the EPM (HC-EPM). Thus, ratio times of HC-EPM rats were compared to the ratio expected by chance exploration of the arms of the EPM (i.e., 0.5). Results indicated that ratio times of HC-EPM rats were indeed below 0.5 suggesting an anxiogenic effect [t(5)=2.89, p<0.04].

4.4.3 pCREB Analysis

4.4.3.1 PAG

The PAG was divided into two approximately equal AP plane ranges, a more anterior range (5.8 mm to 6.28 mm posterior to bregma) and a more posterior range (6.36 mm to 6.84 mm posterior to bregma) (similar to Chapter 3). Relative OD unit data were analyzed with a three-way mixed ANOVA assessing Group, Hemisphere, AP plane with repeated measures on Hemisphere and AP plane. Each column was analyzed separately. Mean contrasts were made using the Tukey-Kramer Multiple-Comparison Test or Bonferroni protected t-tests.

4.4.3.1.1 Handled Control (HC) Versus Handled Control Plus Elevated Plus Maze (HC-EPM)

There were no main Group effects or Group interactions between the two control groups (HC and HC-EPM) in any column of the PAG [all $F(1,10)<2.98$, $p>0.05$]. Therefore, the two control groups [now referred to as combined control (CC)] were combined to simplify the analysis. However, there were AP plane effects in all columns [all $F(1,10)>5.83$, $p<0.05$]. In all three columns, the more posterior AP plane range showed elevated pCREB levels compared to the more anterior AP range (Figure 4.2).

4.4.3.1.2 Predator Stress Plus Elevated Plus Maze (PS-EPM) Versus Combined Controls (CC)

In the dorsal column, there was a three-way interaction of Group x Side x AP Plane ($F(1,16)=10.05$, $p<0.05$). For the anterior AP Plane range (AP Plane 1, Figure 4.3), EPM experience in predator stressed rats increased pCREB-lir in both hemispheres; whereas in the posterior AP range (AP Plane 2, Figure 4.3), EPM experience in predator stressed rats increased pCREB-lir in the right hemisphere only [all Bonferroni $t(16)>4.39$, $p<0.05$]. As reported above, pCREB-lir of controls in the more posterior AP plane exceeded that seen in the more anterior plane. This was also the case in predator stressed rats but only in the right hemisphere (Figure 4.3).

There was a three-way Group x Side x AP plane interaction in the lPAG [$F(1,16)=5.28$, $p<0.05$]. There were no differences in the anterior AP plane between controls (CC) and predator stressed (PS-EPM) rats in either hemisphere. In the posterior

AP plane, however, EPM exposure in predator stressed rats increased pCREB-lir as compared to CC in the right hemisphere only [$t(16)=4.49$, $p<0.05$; Figure 4.3]. In addition, control and predator stressed rats showed higher pCREB-lir in the posterior AP range compared to the anterior AP range [all $t(16)>4.21$, $p<0.001$; Figure 4.3], which suggests that basal pCREB levels are higher in the more posterior AP range of the PAG compared to the anterior AP range in the lateral column. There was a main AP plane effect for the ventral column [$F(1,16)=11.91$, $p<0.05$]. Specifically, more pCREB-lir was expressed in the posterior AP range than the anterior range across all groups, which did not differ in a given AP plane range (Figure 4.3).

4.4.3.2 Amygdala

The amygdala was divided into three AP regions: anterior (1.8 mm posterior to bregma), middle range (2.12 mm to 2.68 mm posterior to bregma) and posterior range (greater than 2.68 mm posterior to bregma). This was done to compare with previous work examining pCREB-lir after predator stress (Chapter 3; Adamec et al., *in press*). Relative OD unit data were analyzed with a three-way mixed ANOVA assessing Group, Hemisphere and Nucleus with repeated measures on Hemisphere and Nucleus separately for each of the three AP plane ranges.

4.4.3.2.1 Handled Control (HC) Versus Handled Control Plus Elevated Plus Maze (HC-EPM)

Similar to the PAG results, there were no Group effects or Group interactions so control groups (HC and HC-EPM) were combined [all $F(5,40)<0.68$, $p>0.05$]. There were Nucleus effects in all AP plane ranges [all $F(5,40)>3.10$, $p<0.05$; Figure 4.4, left panels]. In the anterior AP range, the ACo and BOAT exhibited equally dense staining that was greater than seen in all other nuclei (Tukey-Kramer, $p<0.05$). In the middle AP range, pCREB-lir was highest in the ACo; lowest in the BL, BLv, Ce, and La; and moderate in the BM and Me. pCREB-lir in the PCo fell in between that of the ACo and BM/Me (Tukey-Kramer, $p<0.05$). In the posterior AP range, the ACo, Me and PCo exhibited equally dense pCREB staining that was greater than that seen in all other nuclei which did not differ (Tukey-Kramer, $p<0.05$).

4.4.3.2.2 Predator Stressed Plus Elevated Plus Maze (PS-EPM) Versus Combined Controls (CC)

Relative OD unit data were analyzed with a three-way mixed ANOVA assessing Group, Hemisphere and Amygdala Nucleus with repeated measures on Hemisphere and Nucleus separately for each of the three AP plane ranges. There were only Nucleus effects in the three AP planes (anterior AP plane, $F(5,65)=17.88$, $p<0.05$; middle AP plane, $F(7,110)=28.63$, $p<0.05$; posterior AP plane, $F(7,106)=27.07$, $p<0.05$). In the anterior AP plane, ACo and BAOT showed equal and more pCREB-lir than BLa, Ce, La, and Me (Tukey-Kramer, $p<0.05$; Figure 4.4, right panels). In the middle AP plane range,

ACo and PCo showed the most pCREB-lir followed by BM and Me which together were greater than the remaining nuclei, which did not differ (Tukey-Kramer, $p<0.05$; Figure 4.4, right panels). In the posterior AP range, the ACo, Me and PCo showed equal and increased pCREB-lir as compared with BLa, BLv, BM, Ce, and La, which did not differ (Tukey-Kramer, $p<0.05$; Figure 4.4, right panels). These patterns were like those of the handled controls (Figure 4.4, right panels). Together, the data show that EPM exposure per se or EPM exposure after predator stress was without effect on pCREB-lir in the amygdala.

4.4.3.3 BNST

The BNST was divided into two AP plane ranges, an anterior range (0.26 mm to 0.40 mm posterior to bregma) and a posterior region (0.92 mm posterior to bregma), as was done in Chapter 3. For the anterior range, the BNST was divided into three nuclei: lateral, medial and ventral (according to Paxinos & Watson, 1982). Relative OD unit data were analyzed with a three-way mixed ANOVA assessing Group, Hemisphere and BNST Nucleus with repeated measures on Hemisphere and Nucleus (lateral, ventral, medial and posterior BNST).

4.4.3.3.1 Handled Control (HC) Versus Handled Control Plus Elevated Plus Maze (HC-EPM)

Relative OD unit data were analyzed with a three-way mixed ANOVA. As with the PAG and amygdala, there were no Group effects or Group interactions so control

groups (HC and HC-EPM) were combined (all $F<2.73$, $p>0.05$). There was a main Nucleus effect [$F(3,27)=8.20$, $p<0.05$; Figure 4.5, top panel). The Posterior BNST expressed the most pCREB-lir, ventral expressed the least and medial and lateral fell in between (Tukey-Kramer, $p<0.05$).

4.4.3.3.2 Predator stressed plus elevated plus maze (PS-EPM) versus combined controls (CC)

There was a main Nucleus effect only [$F(3,44)=9.39$, $p<0.001$]. pCREB-lir was highest in the posterior BNST and lateral BNST nucleus, intermediate in the medial BNST nucleus and lowest in the ventral BNST nucleus (Tukey-Kramer, $p<0.05$; Figure 4.5, bottom panel).

4.4.4 PAG Behavior Correlations

Multiple correlations were done to assess whether behavior during the cat exposure predicted changes in pCREB-lir in the PAG. This was done because Adamec et al. (2003) have shown that three behaviors (rat active defense, time immobile, and number of cat bites) during the cat exposure correlate highly with increased pCREB-lir 20-25 minutes after cat exposure in the right lPAG (Adamec et al., 2003). In the present study, rat active defense, time immobile, and number of cat bites did not predict pCREB-lir in either the dorsal or lateral column of the PAG (all $p>0.25$, $df=2$). This suggests that predator stress-induced changes in pCREB-lir in the PAG were *not* sustained one week after predator stress.

In addition, correlations were also done to assess whether anxiety measures in the EPM predicted changes in pCREB-lir in the PAG. Since correlations can only be done in animals exposed to the EPM, multiple comparisons were made between pCREB-lir in HC-EPM and PS-EPM groups. The same pattern of pCREB-lir in the lPAG was found in PS-EPM compared to HC-EPM as was seen in the PS-EPM compared to CC (see Figure 4.3, Tukey Kramer, $p<0.05$). In the dPAG, multiple comparisons showed a similar pattern of pCREB-lir in the PS-EPM compared to the HC-EPM, as was seen in PS-EPM compared to CC (Figure 4.3, Tukey-Kramer, $p<0.05$). However, there was one difference; the predator stress plus EPM-induced increase in pCREB-lir in the right dPAG in AP plane 1 compared to combined controls was lost when the PS-EPM was compared to HC-EPM only (Tukey-Kramer, $p>0.05$).

Two measures of ALB in the EPM, ratio time and ratio entry (measures suggesting increased anxiety), correlated only with the enhanced pCREB-lir in the right lPAG in AP plane 2 of predator stressed rats (ratio time, $r=-.72$, $t(10)=3.30$, $p<0.009$; ratio entry, $r=-.67$, $t(10)=2.34$, $p<0.02$). Reduced ratio time and ratio entry did not significantly correlate with pCREB-lir in any other area in the lPAG. There was also a trend for ratio time and ratio entry to correlate with the elevated pCREB-lir in the right dPAG in AP plane 2 of predator stressed rats (ratio time, $r=-.49$, $t(10)=1.77$, $p<0.054$, 1-tailed; ratio entry, $r=-.47$, $t(10)=1.69$, $p<0.051$, 1-tailed). There was no significant correlation in the right dPAG in AP plane 1 ($p>0.05$). In AP plane 1 in the left dPAG, ratio time did not correlate with pCREB-lir ($p>0.05$) but ratio entry did ($r=-.50$, $t(10)=1.82$, $p<0.049$, 1-tailed). There was no significant correlation between anxiety

measures in the EPM and pCREB-lir in AP plane 1 in the left dPAG ($p>0.05$). These results suggest that some aspect of the rat's response to the EPM, in combination with predator stress, may be driving CREB phosphorylation in the PAG.

4.5 Discussion

Two questions were addressed in this experiment. First, do neuroplastic changes occur after exposure to the EPM per se? Second, is there an enhanced stress sensitization in the neuroplastic response to the EPM in animals previously exposed to a cat? To address these questions, pCREB-lir was examined in the amygdala, PAG and BNST after exposure to the EPM in naïve rats and in rats previously exposed to a cat.

4.5.1 Exposure to the Elevated Plus Maze in Naïve Rats

Exposure to the EPM itself was anxiogenic and stressful as naïve control rats tended to spend more time in the closed arms of the maze than the open arms. This is consistent with the finding that a five minute exposure to the EPM elevates corticosterone (Adamec et al., accepted; File et al., 1994). To examine whether neuroplastic changes occur after exposure to the EPM, we measured pCREB-lir in various brain areas known to be involved in the stress response. We have previously shown that pCREB levels are changed by predator stress (Adamec et al., 2003; Adamec et al., *in press*; Chapter 3). In particular, pCREB-lir is elevated in the right lPAG, suppressed in the BNST, and increased or reduced in various amygdala nuclei, depending on hemisphere and AP plane (Chapter 3). Although exposure to the EPM is stressful, we found no differences in

pCREB levels between control groups (Handled only and Handled plus EPM) in all brain areas investigated. This suggests that exposure to the EPM per se does not engage neuroplastic changes, at least those related to pCREB, in brain areas known to be involved in the stress experience. This is consistent with Hebda-Bauer et al. (2004) who found that $CREB^{\alpha\delta-}$ deficient mice show no changes in ALB as measured in the EPM. Yet, Wallace et al. (2004) found that rats made to over-express CREB in the BLa are more anxious. CREB deficient mice carry the deficiency from birth, whereas Wallace et al. (2004) induced CREB changes in normal adult rats. In our study, however, we assessed pCREB levels, not alterations in CREB expression. This is an important distinction since changes in pCREB-lir may occur independently of changes in total CREB levels, as observed by Bilang-Bleuel et al. (2002) after swim stress.

In addition to pCREB, c-*fos* has been examined as a marker of neural activity in brain areas after exposure to the EPM. c-*fos* is an immediate early gene (IEG) product that is inducible via cyclic AMP response element (CRE)-mediated transcriptional activation (Herrera & Robertson, 1996; Herdegen & Leah, 1998; Morgan & Curran, 1991). Several studies have shown c-*fos* elevation in a variety of brain structures, including the amygdala and PAG, after exposure to the EPM (Duncan, Knapp, & Breese, 1996; Hinks, Brown, Field, Poat, & Hughes, 1996; Silveira, Sandner, & Graeff, 1993; Silveira, Zangrossi, de Barros, Viana, Silveira, & Graeff, 2001). In the present study however, there was no evidence of elevated pCREB-lir in rats exposed to the EPM in the amygdala or PAG. Importantly, although pCREB can activate c-*fos* through CRE activation, lack of changes in pCREB suggests that the increases in c-*fos* after exposure

to the EPM are not transduced via pCREB-dependent pathways. This is consistent with a number of studies that have shown different patterns of change in pCREB- and c-*fos*-lir after stress (Gammie & Nelson, 2001; Stanciu, et al., 2001).

4.5.2 Exposure to the Elevated Plus Maze in Predator Stressed Rats

4.5.2.1 Changes in Behavior

Predator stress lastingly increases ALB in the EPM. This is consistent with several studies showing potentiated ALB in the EPM in animals with prior exposure to a variety of stressors, such as social defeat, forced swim, inescapable shock and predator stress (Adamec & Shallow, 1993, Blundell et al., 2005 - Chapter 2; Heinrichs, Menzaghi, Pich, Baldwin, Rassnick, Britton, Koob, 1994; Heinrichs, Pich, Miczek, Britton, Koob, 1992; Korte, De Boer, De Kloet, Bohus, 1995; Roozendaal, Bohus, & McGaugh, 1996). As reported often in the past (Adamec, 1997; Adamec et al., 2001; Adamec & Shallow, 1993; Adamec et al., 1997; Blundell et al., 2005 - Chapter 2), the increase in EPM anxiety cannot be accounted for by changes in exploratory behavior or activity.

4.5.2.2 Changes in pCREB-lir

Since predator stress lastingly potentiates ALB in the EPM, we expected to see changes in pCREB-lir in brain areas associated with fear following EPM exposure in predator stressed rats relative to controls. Changes in pCREB-lir have been reported in all amygdala nuclei, the right lPAG and most BNST nuclei (Adamec et al., 2003; Adamec et al., *in press*; Chapter 3) 20-25 minutes after predator stress. In the present

study, seven days after predator stress, we observed increases in pCREB-lir only in the PAG following exposure to the EPM in predator stressed rats. Specifically, pCREB-lir was elevated in both hemispheres in the anterior AP plane of the dPAG and increased in the right dPAG in the posterior AP plane range after EPM in predator stressed rats compared to controls (Combined Controls). The pCREB changes in dPAG are of particular interest since predator stress alone does not alter pCREB-lir in this column (Adamec et al., 2003; Chapter 3). This suggests that a combination of predator stress followed by exposure to the EPM may be required to activate the dPAG. pCREB-lir was also elevated in the posterior AP plane range of the right lPAG after exposure to the EPM in predator stressed rats compared to controls (Combined Controls). This is consistent with previous research in which predator stress elevated pCREB-lir in the right lPAG (Adamec et al., 2003; Chapter 3). It is important to emphasize that EPM testing occurred seven days after cat exposure suggesting a long-lasting stress susceptibility to EPM induction of pCREB in predator stressed rats. This indicates that relatively mild stressors may further engage mechanisms associated with neuroplasticity in people with a history of severe stress. If this occurs, neural mechanisms of prior traumatic stress may interact with subsequent stress to reinforce psychopathology. This may have important ramifications for PTSD in that there is an exaggerated reaction to mild stressors in which the response is more appropriate to the original traumatic situation than the current conditions (Bremner et al., 1995; Dykman et al., 1997; Friedman, 1994).

Since pCREB-lir is enhanced in the right lPAG after predator stress, one possible explanation for the elevated pCREB-lir after EPM in predator stressed rats is that the

pCREB changes are sustained from the time of the predator stress exposure. Although it was not directly tested in this study, it does not appear to be the case for several reasons. First, Adamec et al. (2003) have previously shown that behaviors during the cat exposure, including rat active defenses, time immobile, and number of cat bites, strongly predict predator stress-induced increases in pCREB-lir in the right lPAG. In the present study, however, there was no correlation between the three behaviors in the cat exposure and elevated pCREB-lir in either the right lPAG or the dPAG. Second, changes in pCREB-lir in the dPAG were not seen in predator stressed animals only (Adamec et al., 2003; Chapter 3). Third, there is evidence for the EPM "experience" driving pCREB-lir. For instance, ALB in the EPM, measured as decreased ratio time and ratio entry, predicted elevated pCREB-lir in the right lPAG in AP plane two. Similarly, although not as strong, ALB in the EPM tended to predict elevated pCREB-lir in the dPAG. This suggests that enhanced pCREB-lir may be dependent on an interaction between the prior predator stress experience and exposure to the milder EPM stressor. However, future studies that examine pCREB one week after predator stress only are necessary to support this statement.

It is somewhat surprising, however; that changes in pCREB-lir were not induced in the amygdala after EPM exposure in predator stressed rats. pCREB activation, or inactivation, in the amygdala has been found after exposure to the EPM in previously stressed rats. In particular, Pandey et al., (2003) found decreased pCREB-lir in the Ce and Me amygdala in animals exposed to the EPM after ethanol withdrawal. Furthermore, c-*fos* expression is enhanced in the amygdala after exposure to the EPM in rats previously

given an anxiogenic drug (Troakes & Ingram, 2005). In our study, however, we found no differences in pCREB-lir in the amygdala after EPM in animals previously exposed to a cat compared to controls. One of the key differences between the studies discussed above and the present study is that in our study, predator stress occurred seven days prior to EPM exposure. In the other two studies, stress was induced less then 24 hours prior to EPM exposure (Pandey et al., 2003; Troakes & Ingram, 2005). Furthermore, as discussed above, changes in pCREB-lir do not necessarily mimic changes in c-*fos*. Finally, all three studies (present study included) used different methods to induce stress/fear.

Amygdala activation may require that the stressors be closer together in time and/or stronger. Perhaps the mild stress produced by the EPM was not enough to alter pCREB-lir in rats that were previously exposed to a cat. Experiments that shorten the time interval between predator stress and exposure to the EPM and studies using multiple stressors (cat exposures) may result in amygdala activation, as measured by changes in pCREB. Lack of activation in the amygdala in response to the EPM in predator stressed rats will be discussed further in the following section.

4.5.3 Potential Role of NMDA Receptors in Predator Stressed Rats Exposed to Elevated Plus Maze

Changes in pCREB-lir in the PAG after exposure to the EPM were specific to animals with prior exposure to a stressor. This suggests that prior stress may cause a drop in threshold for pCREB activation. Importantly, this drop in threshold is specific to

the PAG. One plausible explanation for increased pCREB-lir in the PAG after EPM in predator stressed rats is as follows. CREB phosphorylation in the PAG may be a "barometer" for tone or state of responsiveness of the fear circuit. PAG activation by EPM exposure likely involves glutamate action at NMDA receptors because NMDA agonists and antagonists injected into the PAG cause anxiogenic and anxiolytic behaviors respectively, in the EPM (Carobrez, Teixeira, & Graeff, 2001; Guimaraes, Carobrez, de Aguiar, Graeff, 1991; Molchanv & Guimaraes, 2002). Responsiveness of a PAG NMDA receptor-dependent anxiety promoting system may be lastingly increased by LTP-like changes in the right Ce-lPAG pathway known to be produced by predator stress (Adamec et al., 2001; Adamec et al., 2005a). Support for this idea arises from several observations. First, predator stress is lastingly anxiogenic in the EPM and equally lastingly potentiates Ce-lPAG neural transmission, both in an NMDA receptor-dependent manner (Adamec et al., 1999a; Adamec et al., 2005b). Second, path analysis supports a model whereby enhanced right Ce-lPAG transmission in part drives enhanced EPM anxiety. This suggests that exposure to the EPM activates the Ce-lPAG pathway and if the pathway is potentiated by prior predator stress, enhanced anxiety is observed. Under this hypothesis, in naïve rats, EPM exposure produces little CREB phosphorylation via glutamate/NMDA receptor activation because the NMDA/pCREB system is only slightly engaged. In rats with a history of predator stress and Ce-lPAG LTP, however, EPM activation of the NMDA/pCREB system is enhanced sufficiently to induce further pCREB-lir. These ideas require further experimentation to test their validity. If this model is correct, an NMDA receptor antagonist given prior to exposure to the EPM in

predator stressed rats should block the anxiogenic response in the EPM and block the elevated pCREB levels in the PAG.

One implication is that the EPM experience, in combination with predator stress, may further potentiate Ce-lPAG LTP. Since pCREB changes were also seen in the dPAG, exposure to the EPM in predator stressed rats may also potentiate transmission from the Ce to the dPAG, but perhaps to a lesser extent. Results of the present study suggest that pCREB activation may be stronger in the lPAG than the dPAG for the following reasons. First, monosynaptic projections from the Ce to the dPAG are less than projections from Ce to the lPAG (Davis, M. personal communication). Second, pCREB-lir in the right lPAG correlated more strongly with ALB in the EPM than that in the dPAG.

This model might also predict altered pCREB-lir in the amygdala (Ce), which was not seen in the present study. One plausible reason is that LTP-like changes in the Ce-lPAG pathway appear to be postsynaptic (Adamec et al., 2001) which suggests that EPM-induced NMDA receptor-dependent changes in pCREB-lir should only occur in the PAG, and not in the Ce.

From a stress perspective, increased glutamate release to the PAG may be a mechanism for strengthening of affect change following severe stress to subsequent stressors. Importantly, alterations of glutamatergic and NMDA receptor functions have been proposed as part of the etiology of PTSD in humans (Van der Kolk, 1994).

4.5.4 Conclusions

Exposure to a mild stressor (EPM) does not induce neuroplastic changes, as measured by changes in pCREB, in brain areas associated with fear and anxiety. In contrast, prior predator stress produces an enhanced pCREB response to the EPM. Since cat exposure (predator stress) occurred seven days prior to EPM testing, this suggests a lasting stress susceptibility has been induced by cat exposure. Finally, the PAG (i.e., the dorsal and lateral columns) appear to be involved in the stress sensitization.

Figure Captions

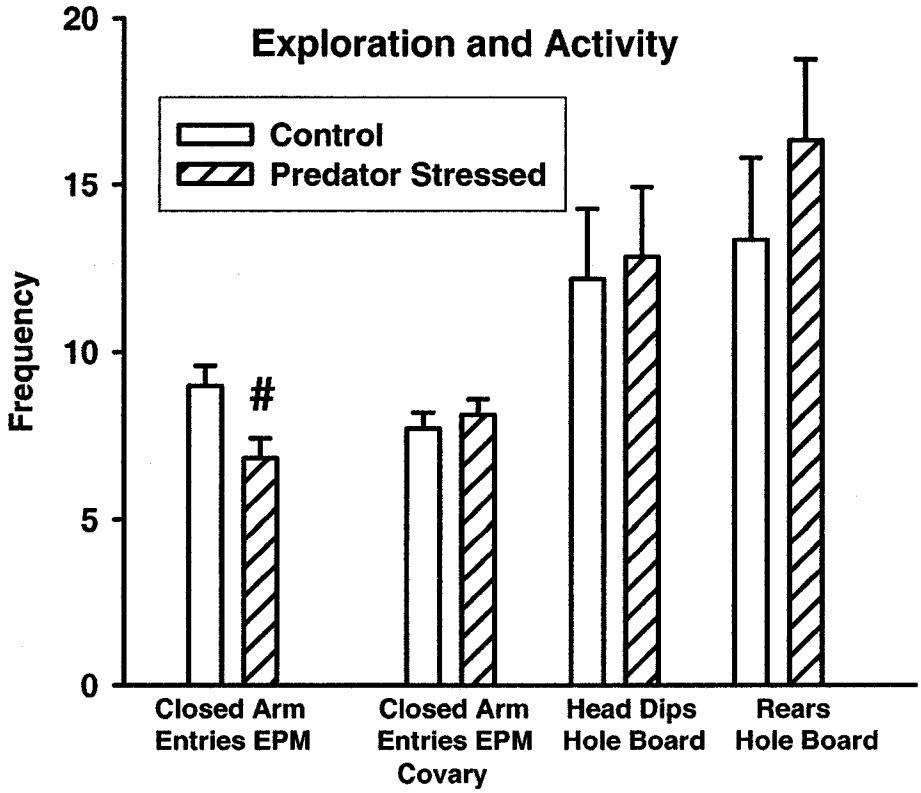
Figure 4.1. Plotted are Mean + SEM of Hole Board and EPM behaviors of controls and predator stressed rats. Means marked with a # differ from control ($p<0.05$). Top Panel: The two left most panels show frequency of closed arm entries in EPM as original data or after covarying ratio time and ratio entry ('covary'). The two right plots show frequency of head dips and rears in the Hole Board test. Bottom Panel: the three left most plots are referenced to the left ordinate, while unprotected head dips are referenced to the right ordinate.

Figure 4.2. Mean + SEM relative optical density units (PAG optical density units divided by total section optical density units) in all three columns for both groups [Handled control (Handled no EPM), Handled control plus EPM (Handled and EPM)] are graphed. For a given column plot, means marked with the same letter do not differ but differ from those with different letters (Tukey-Kramer tests, $p<0.05$).

Figure 4.3. Mean + SEM relative optical density units (PAG optical density units divided by total section optical density units) in all three columns for both groups (combined controls and predator stressed) are graphed. Means marked with a # differ from their corresponding controls. Means in AP plane 1 marked with a 2 differ from the same mean in AP plane 2 (Tukey-Kramer tests, $p<0.05$). In the bottom panel, means marked with the same letter do not differ but differ from those with different letters (Tukey-Kramer tests, $p<0.05$).

Figure 4.4. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units) in all nuclei between groups [Handled control (Handled no EPM, H), Handled control plus EPM (Handled and EPM, M)] are graphed in the left panels. Means marked with the same letter do not differ but differ from those with different letters (Tukey-Kramer, $p<0.05$). H-handled only, M-handled plus EPM. In the right panels Mean + SEM relative optical density units in all nuclei collapsed over Hemisphere and all Groups are graphed. Means marked with the same letter do not differ but differ from those with different letters (Tukey-Kramer, $p<0.05$). Me – medial nucleus; ACo – anterior cortical nucleus; BM – basomedial nucleus; BLa – basolateral nucleus; La – lateral amygdala; Ce – central nucleus, BAOT - bed nucleus accessory olfactory tract, BLv - basolateral ventral.

Figure 4.5. In the top panel, Mean + SEM relative optical density units (BNST optical density units divided by whole section optical density units) in all nuclei (anterior BNST - Medial, Lateral, Ventral, & Posterior BNST) between groups [Handled control (Handled no EPM), Handled control plus EPM (Handled plus EPM)] are graphed. Means marked with the same letter do not differ but differ from those with different letters (Tukey-Kramer, $p<0.05$). In the bottom panel, Mean + SEM relative optical density units (BNST optical density units divided by whole section optical density units) in all nuclei collapsed over Hemisphere and Groups are graphed. Means marked with the same letter do not differ but differ from those with different letters (Tukey-Kramer, $p<0.05$).


Exploration and Activity
Control
Predator Stressed
Frequency
20
15
10
5
0
#
Closed Arm Entries EPM
Closed Arm Entries EPM Covary
Head Dips Hole Board
Rears Hole Board

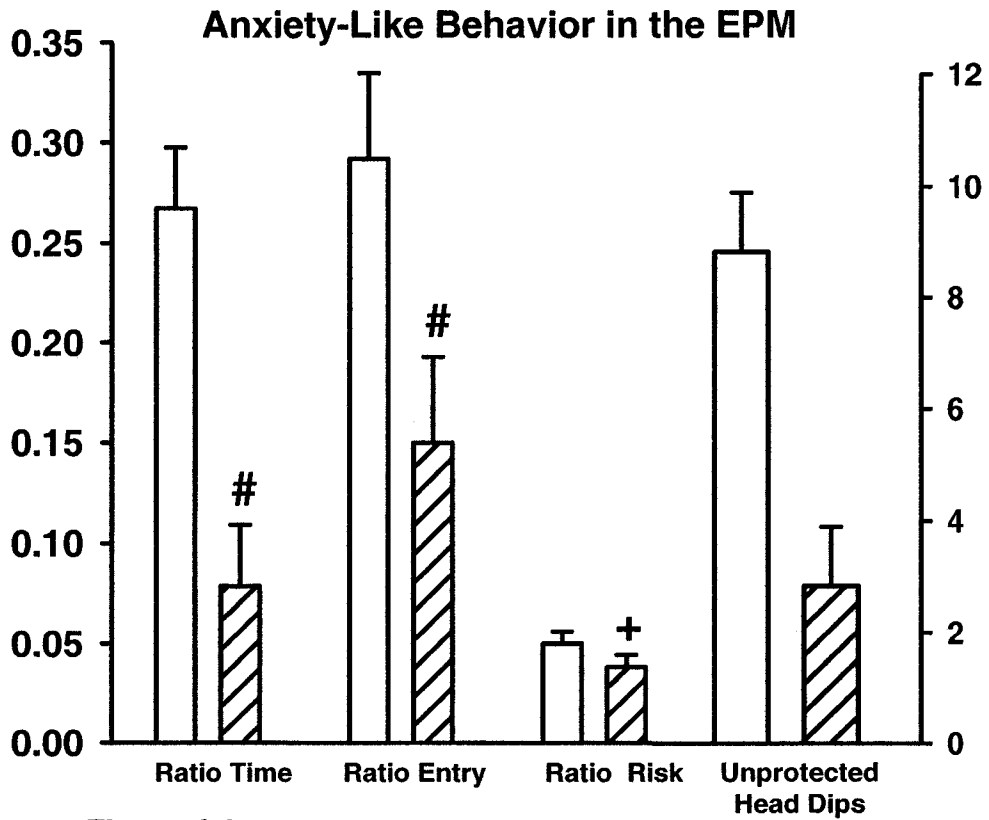

Anxiety-Like Behavior in the EPM
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
12
10
8
6
4
2
0
#
#
+
Ratio Time
Ratio Entry
Ratio Risk
Unprotected Head Dips

Figure 4.1

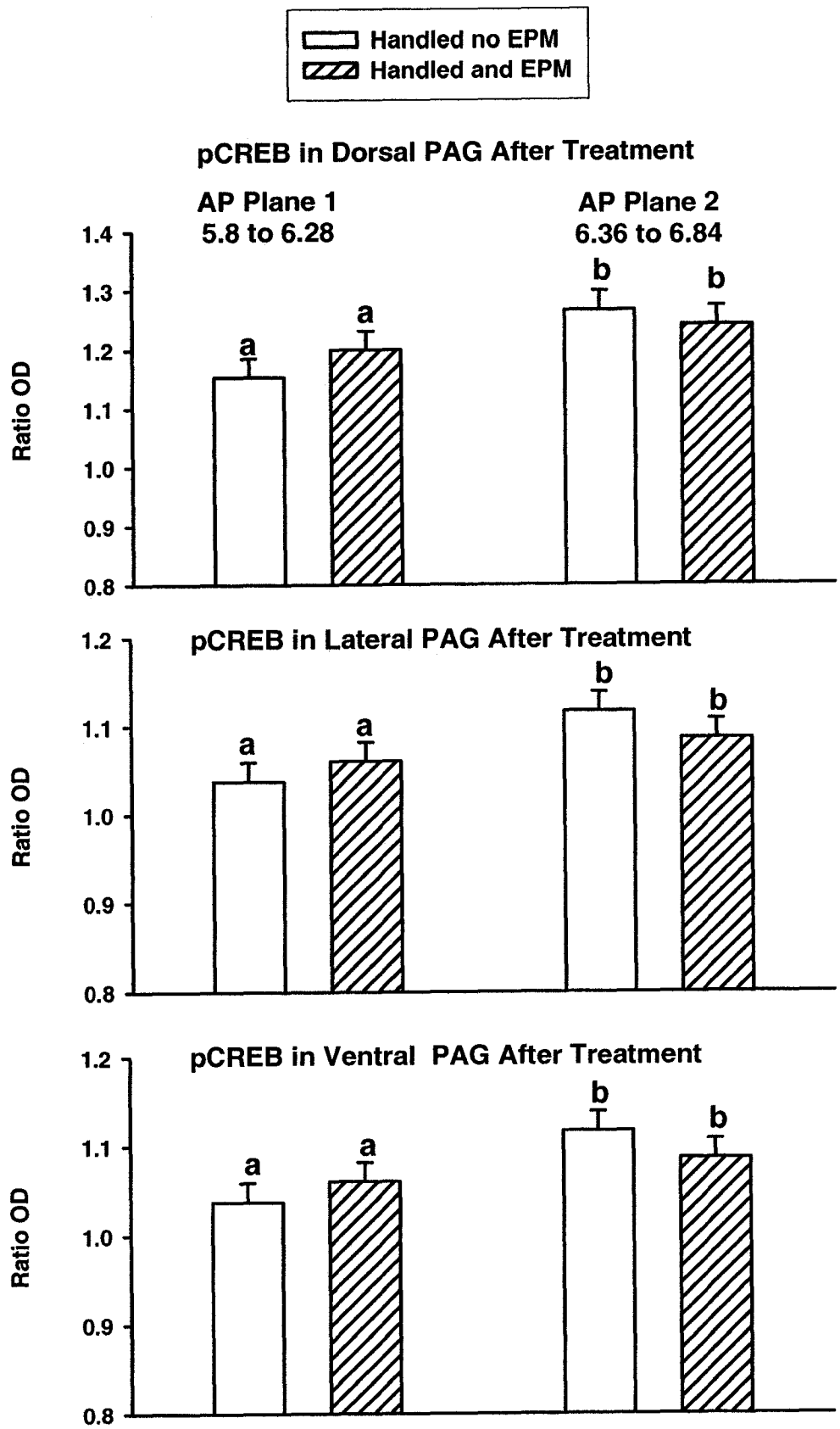

Handled no EPM
Handled and EPM
pCREB in Dorsal PAG After Treatment
AP Plane 1
5.8 to 6.28
AP Plane 2
6.36 to 6.84
Ratio OD
1.4
1.3
1.2
1.1
1.0
0.9
0.8
a
a
b
b
pCREB in Lateral PAG After Treatment
Ratio OD
1.2
1.1
1.0
0.9
0.8
a
a
b
b
pCREB in Ventral PAG After Treatment
Ratio OD
1.2
1.1
1.0
0.9
0.8
a
a
b
b

Figure 4.2

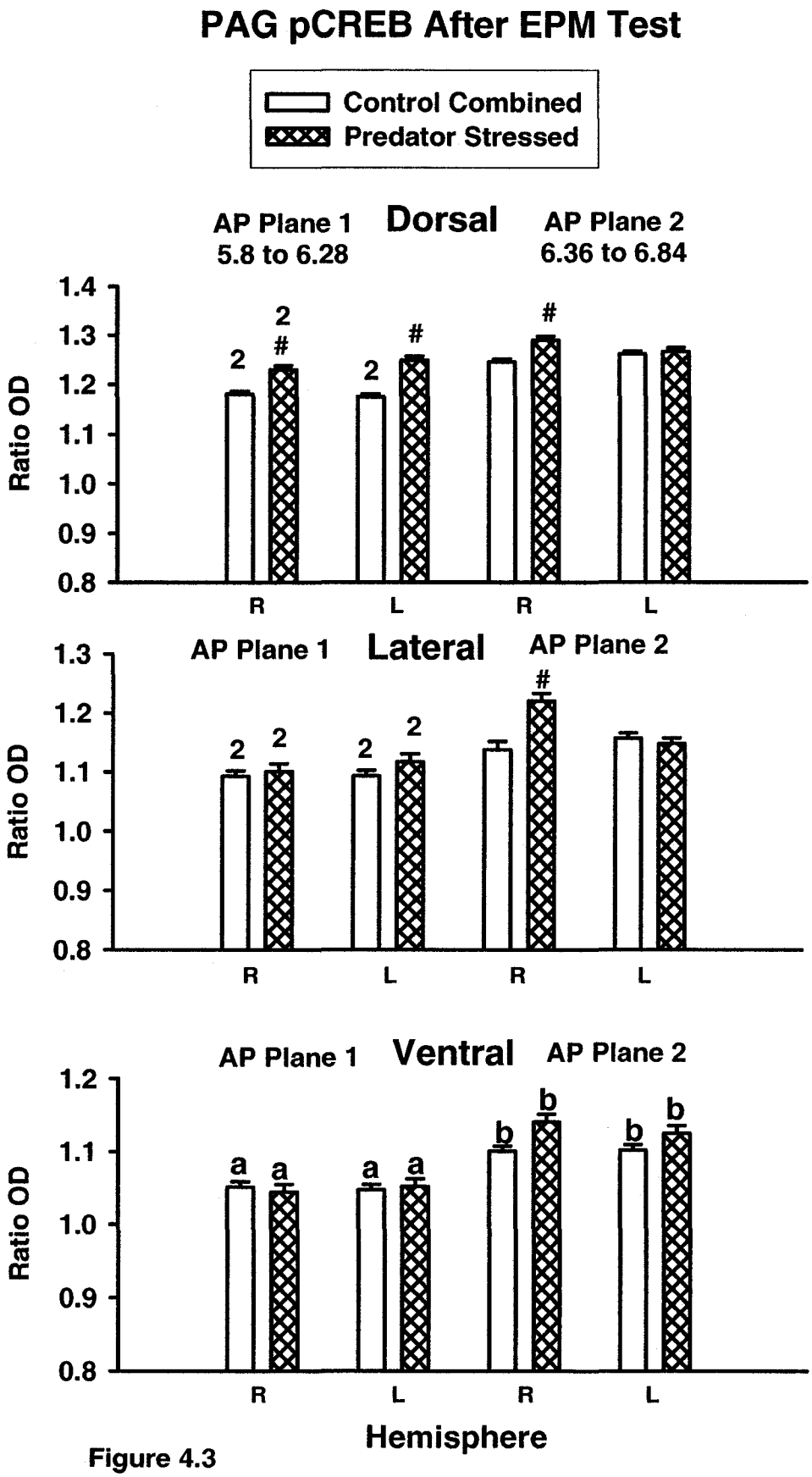

PAG pCREB After EPM Test
Control Combined
Predator Stressed
AP Plane 1
5.8 to 6.28
Dorsal
AP Plane 2
6.36 to 6.84
Ratio OD
1.4
1.3
1.2
1.1
1.0
0.9
0.8
R
L
R
L
AP Plane 1
Lateral
AP Plane 2
Ratio OD
1.3
1.2
1.1
1.0
0.9
0.8
R
L
R
L
AP Plane 1
Ventral
AP Plane 2
Ratio OD
1.2
1.1
1.0
0.9
0.8
R
L
R
L
Hemisphere

Figure 4.3

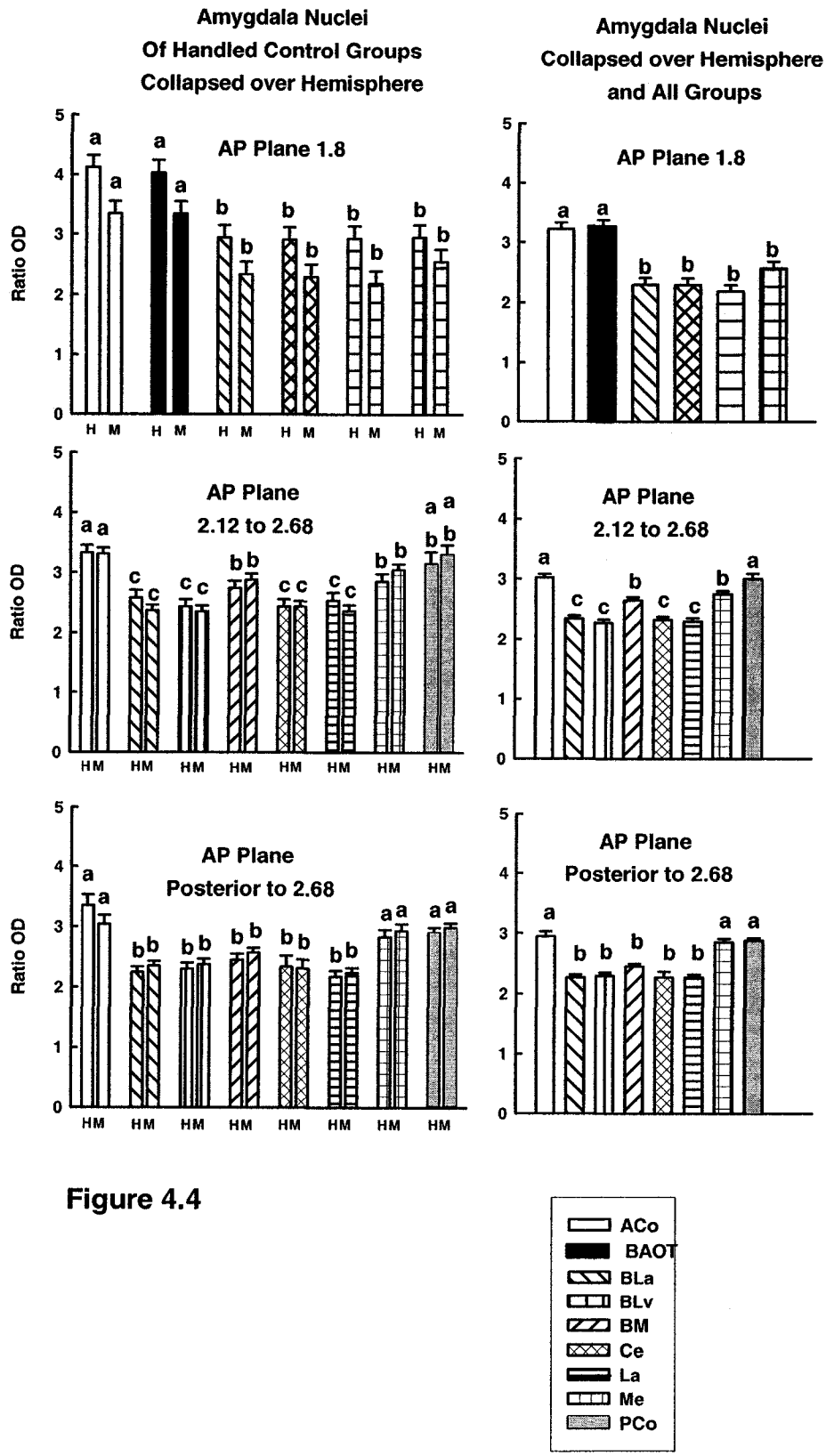
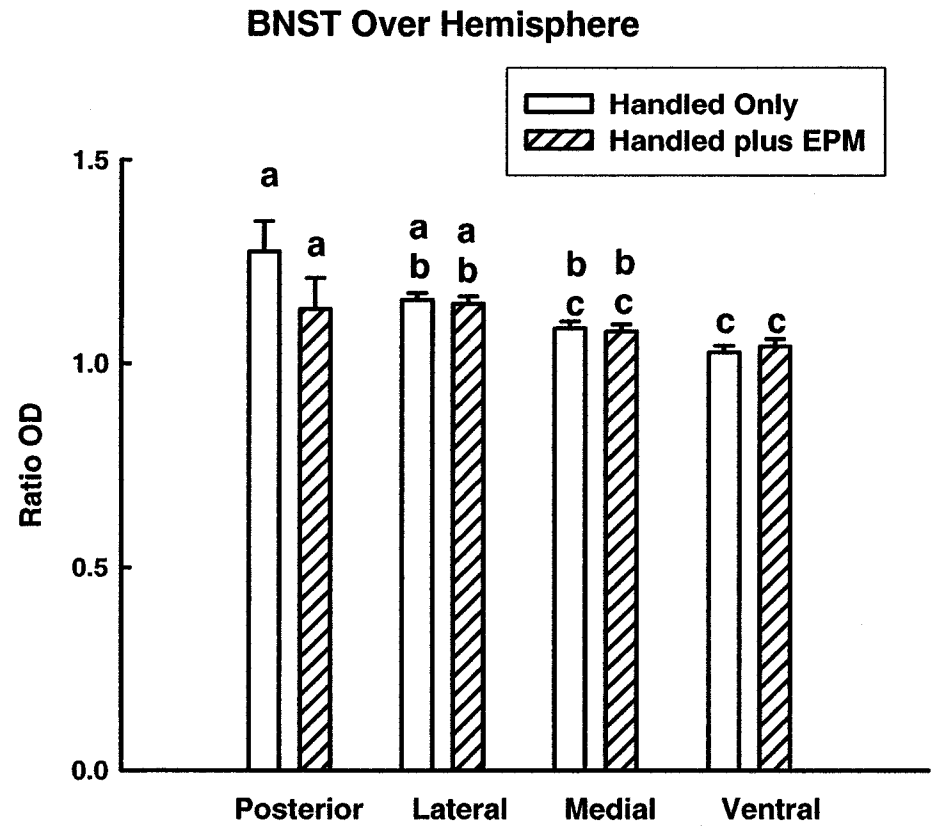

Amygdala Nuclei
Of Handled Control Groups
Collapsed over Hemisphere
Amygdala Nuclei
Collapsed over Hemisphere
and All Groups
AP Plane 1.8
AP Plane 1.8
AP Plane
2.12 to 2.68
AP Plane
2.12 to 2.68
AP Plane
Posterior to 2.68
AP Plane
Posterior to 2.68
Ratio OD
H M
HM
ACo
BAOT
BLa
BLv
BM
Ce
La
Me
PCo

Figure 4.4


BNST Over Hemisphere
Handled Only
Handled plus EPM
Ratio OD
1.5
1.0
0.5
0.0
a
a
a b
a b
b c
b c
c
c
Posterior
Lateral
Medial
Ventral

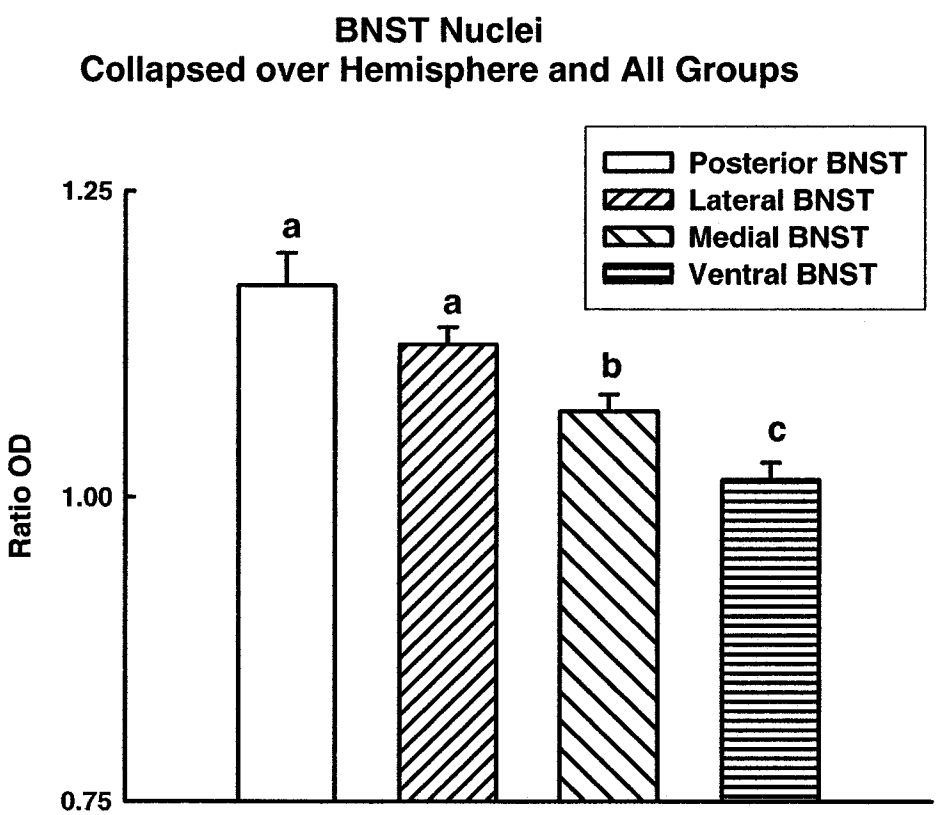

BNST Nuclei
Collapsed over Hemisphere and All Groups
Posterior BNST
Lateral BNST
Medial BNST
Ventral BNST
Ratio OD
1.25
1.00
0.75
a
a
b
c

Figure 4.5

Chapter 5: Summary

The main goal of this dissertation was to better understand the brain and behavioral changes that occur as a result of exposure to traumatic stressor(s). This is a relevant topic as approximately 15% of people who experience a stressful event, develop PTSD (Kessler et al., 1995). There is no cure for this disorder; thus, understanding the impact of stress on brain function is an important area of research.

In this dissertation, I employed the ecologically valid predator stress model to assess the effects of stress on brain and behavior. Predator stress is both fear provoking and stressful (Adamec et al., 1998a; Blanchard et al., 1998; Dielenberg et al., 2001a; McGregor et al., 2002). In particular, cat exposure elicits immediate behavioral and endocrine responses and induces long-lasting changes in defense and ALB (Adamec & Shallow, 1993; Adamec et al., 2001; Cohen et al., 2004). Using this model, I addressed four main questions. First, are the anxiogenic effects of predator stress NMDA receptor-dependent? Second, are the neuroplastic changes that occur after predator stress NMDA receptor-dependent? Third, do neuroplastic changes occur after exposure to other, milder stressors, such as the elevated plus maze (EPM)? Finally, is there an enhanced stress sensitization in the neuroplastic response to a mild stressor in animals previously stressed? Each of these questions was addressed in the bodies of the individual chapters. This Summary will begin with a brief overview of the main conclusions from each chapter, followed by a discussion of the role of NMDA receptors in stress, as well as a

brief comment on pCREB analyses techniques. Within each section, I will discuss future research that may be necessary to fully understand the results.

5.1 Overall Conclusions from Studies

As described in Chapter 2, we examined the behavioral effects of an NMDA receptor antagonist (CPP) on predator stress-induced ALB. Consistent with previous studies (Adamec et al., 1999a; Adamec et al., 1999b), most predator stress-induced increases in ALB were blocked with CPP. In particular, CPP blocked the predator stress-induced reduction in open arm exploration and risk assessment as measured in the EPM, it blocked the predator stress-induced decrease in entries into the lighted arm of the light/dark box, and blocked the predator stress-induced prolongation of startle habituation. Behavioral effects of predator stress that were not blocked by CPP included reduction in unprotected head dips in the EPM and suppressed social interaction These findings are consistent with previous research that has shown NMDA receptor-dependent predator stress-induced changes in the EPM (Adamec et al., 1999a). Novel findings include NMDA receptor dependence of predator stress effects on light/dark box behavior and startle habituation. As well, predator stress did not affect ALB as measured by social avoidance and peak startle amplitude. Taken together, the findings add to a body of evidence showing that a lasting syndrome of anxiety-like behavioral changes follows predator stress. Furthermore, components of this syndrome of changes in ALB likely depend on changes in separable neural substrates initiated, in part, by NMDA receptors as well as by other neurochemical means.

The second study, described in Chapter 3, examined the effects of CPP on predator stress-induced changes in pCREB-lir. CPP blocked the predator stress-induced

increase in pCREB-lir in the right lPAG; blocked, enhanced or had no effect on the predator stress-induced changes in pCREB-lir in all amygdala nuclei; reversed the predator stress-induced suppression of pCREB-lir in the BNST; and had no effect on pCREB-lir in the DMH or ACC. Importantly, changes in CREB phosphorylation after predator stress (or CPP plus predator stress) in all amygdala nuclei and the PAG were hemisphere- and AP plane-dependent. Results showed that several amygdala nuclei, the right lPAG and BNST areas, where predator stress changes pCREB-lir in an NMDA receptor-dependent manner, are candidate areas of neuroplastic change contributing to lasting changes in ALB. Finally, consistent with previous results from the amygdala and PAG (Adamec et al., 2003), predator stress increased the amount of pCREB-lir within each PAG cell, but did not increase the number of PAG cells stained for pCREB. Thus, combining both densitometry and cell counting may yield a more complete picture of the nature of pCREB changes.

In Chapter 4, we questioned whether other, milder stressors (i.e., EPM) activated pCREB-lir similarly to predator stress. In addition, since predator stress induces long-lasting increases in ALB as measured in the EPM, we examined whether there was an enhanced stress sensitization in the neuroplastic response to the EPM in animals previously exposed to a cat. Results showed that exposure to the EPM alone did not induce neuroplastic changes, as measured by changes in pCREB-lir, in brain areas associated with fear and anxiety. In contrast, prior predator stress produced an enhanced pCREB response in the dPAG and the right lPAG after exposure to the EPM. Furthermore, since predator stress occurred seven days prior to EPM testing, this suggests

that a long-lasting stress susceptibility was induced by cat exposure. This is particularly important as a key feature of PTSD is the exaggerated reaction to a mild stressor in which the response is more appropriate to the original traumatic situation than the current conditions (Bremner, Krystal, Southwick, & Charney, 1995; Dykman, Ackerman, & Newton, 1997; Friedman, 1994).

5.2 Role of NMDA Receptors in Predator Stress

Due to the nature of the results, many of the studies proposed in the following sections involve manipulation of CREB/pCREB and its effects on behavior. In particular, I have proposed several studies that assess the effects of CREB over-expression on ALB. Importantly, previous research has found increased pCREB in brain areas where CREB levels have been elevated (using viral vectoring methodology similar to Wallace et al., 2004), at least in an odor preference learning paradigm (Yuan, Harley, Darby-King, Neve, & McLean, 2003). This suggests that CREB over-expression-induced increases in pCREB levels may be similar to elevations in pCREB produced by predator stress. In addition to viral mediated gene transfer, acute manipulations of pCREB can be accomplished using a PKA inhibitor. For instance, Pandey et al. (2003) have shown that microinfusing a PKA inhibitor into the Ce (but not the BLa) reduces pCREB-lir and also increases ALB as measured in the EPM. Thus, preliminary studies are required to ensure that predator stress-induced alterations in pCREB can be blocked with a PKA inhibitor.

Another important point to consider regarding CREB over-expression and pCREB reduction is the nature of CREB/pCREB effects on behavior. For instance, Wallace et al. (2004) have shown that increased ALB as measured in the EPM occurs during peak CREB levels (3-4 days after injection). CREB levels return to baseline about one week after it is injected (Carlezon, Boundy, Haile, Lane, Kalb, Neve, Nestler, 1997). The issue remains whether this increase in CREB/pCREB alters ALB when the behavior is measured sometime after the increase in CREB/pCREB expression (i.e., one week). At issue is the question: are lasting changes in ALB a result of sustained CREB/pCREB activation or are they the result of transient CREB/pCREB levels that lead to long-lasting neuroplastic change that in turn, mediate the behavioral changes, or is it a combination of both? To address this issue, preliminary studies must be completed that measure ALB at time intervals other than during peak CREB levels (if using the viral vectoring methodology), for example, one week after peak CREB levels. If increased ALB is still observed and CREB levels are at baseline, it lends support to the theory that transient CREB/pCREB alterations induce long-lasting neuroplastic changes that mediate the increased ALB. Although this was not directly tested in the study discussed in Chapter 4, there are several key points from our data that suggest that pCREB-lir changes after exposure to the EPM in predator stressed rats are not sustained from the cat exposure (see Chapter 4 for full discussion). Of course, to fully address this issue, studies need to be completed which assess pCREB-lir after predator stress (i.e., one week after stress). A second way to test this question is to infuse a PKA facilitator (methods similar to Pandey et al., 2003) and measure ALB and pCREB-lir one week-post injection. Again, if changes

in ALB are mediated by transient changes in pCREB, ALB should be increased and pCREB levels should resemble that of controls (baseline). On the other hand, if increased ALB depends on sustained pCREB/CREB alterations, both ALB as well as pCREB levels should be increased when tested one week later. These are important considerations that must be taken into account in the design of future studies as discussed in the following sections.

5.2.1 Behavioral Changes After Predator Stress - Relationship to Amygdala Afferent and Efferent Transmission

As discussed above, predator stress produces a long-lasting increase in rat ALB (Adamec & Shallow, 1993; Adamec et al., 2003; Blundell et al., 2005 - Chapter 2; Cohen, Joseph, & Matar, 2003). However, inconsistent results have been found across studies. For example, previous work indicates that predator stress facilitates peak startle amplitude (Adamec, 1997). In the study described in Chapter 2, however, there were no effects of predator stress on startle amplitude. We have recently found (in preparation) that more robust and reliable increases in startle amplitude are achieved with a 10 minute cat exposure, rather than the five minute exposure used in these studies. This may also help explain why there were no changes in social avoidance as measured in the Haller test. Therefore, further studies are required to assess the effects of CPP on ALB after a 10 minute cat exposure.

In addition to the behavioral changes, electrophysiological studies in rodents suggest that predator stress produces a potentiation in transmission in amygdala afferent

and efferent pathways. In particular, predator stress-induces LTP-like changes in neural transmission from the Ce to the lPAG and from the VAB to the BLa (Adamec et al., 2001; Adamec et al., 2003; Adamec et al., 2005a). Importantly, this potentiation in the right hemisphere is blocked by a systemic injection of an NMDA receptor antagonist (CPP) prior to predator stress (Adamec et al., 2005b). In addition, amygdala afferent and efferent LTP-like changes are highly predictive of severity of change in ALB following predator stress and LTP-like changes in these pathways in the right hemisphere have been proposed as a mechanism mediating stress-induced changes in ALB (Adamec et al., 2003; Adamec et al., 2005a). Moreover, like stress-induced LTP-like changes in amygdala afferent and efferent neural transmission, most changes in ALB following stress are NMDA receptor-dependent. For instance, systemic administration of both competitive and non-competitive NMDA receptor antagonists before, but not after predator stress prevent lasting increases in ALB (Adamec et al., 1999a; Blundell et al., 2005 - Chapter 2). However, not all predator stress-induced changes in ALB are NMDA receptor-dependent. For example, social interaction is not affected by prior injection of CPP (Blundell et al., 2005 - Chapter 2). This is particularly interesting, as predator stress-induced increases in pCREB-lir in several amygdala nuclei (mid ACo, left BM, left BLa and left BLv) are also not NMDA receptor-dependent (Chapter 3). Perhaps those brain areas that show elevation in pCREB-lir, which are not NMDA receptor-dependent, mediate changes in ALB that are also not NMDA receptor-dependent (i.e., predator stress-induced changes in social interaction). To test this hypothesis, rats could be made to over-express CREB (via viral vector of CREB) or pCREB (via an injection of a PKA

facilitator) in the above mentioned nuclei and then exposed to the social interaction test. As discussed above, both acute and possible sustained effects of these manipulations should be investigated. If changes in ALB are mediated by changes in CREB in those particular nuclei, control rats over-expressing CREB should show increased ALB in the social interaction test (similar to that of predator stressed animals).

Interestingly, benoxathian, an α_1 noradrenergic antagonist, blocks the acute immobilization stress-induced increase in ALB as measured in the social interaction test but does not alter ALB as measured in the EPM (Cecchi, Khoshbouei, & Morilak, 2002). In our laboratory, propranolol, a β-adrenergic antagonist, has been shown to block some anxiety-like behavior changes produced by predator stress (Mathew Grimes, honours thesis). These findings, as well as results from the Chapter 2, suggest that predator stress-induced changes in ALB as measured in the social interaction test which are not NMDA receptor-dependent may be dependent on the noradrenergic system. This is further evidence that the social interaction test and the EPM measure different aspects of behavioral stress reactivity and thus, can be modulated independently. Further studies involving noradrenergic modulation prior to predator stress are necessary to test this hypothesis.

5.2.2 Changes in pCREB-lir After Predator Stress - Relationship to ALB and Amygdala Afferent and Efferent Transmission

In addition to the increases in ALB and changes in amygdala afferent and efferent neural transmission, alterations in pCREB-lir have been found following

predator stress (Adamec et al., 2003; Adamec et al., *in press*; Chapter 3). In the present studies, CPP blocked (or reversed) the predator stress-induced changes in pCREB-lir in particular brain areas (right lPAG, several amygdala nuclei, and the BNST).

The patterns of results have at least three implications. First, changes in pCREB-lir are AP plane- and hemisphere-dependent, at least in the amygdala and PAG. For example, results from Chapter 3 show that pCREB-lir in a particular amygdala nucleus can be increased, suppressed, or unchanged depending on hemisphere and AP plane position after predator stress. This is particularly important as most studies examine pCREB-lir bilaterally in one AP plane. In addition to alterations in pCREB-lir, behavioral effects of limbic sensitization produced by kindling also show hemispheric asymmetries. For example, kindling left BLa is anxiolytic, whereas kindling right BLa is anxiogenic (Adamec et al., 1994; Adamec et al., 2004). Importantly, hemispheric differences in the amygdala have also been found in human PTSD patients (Shin et al., 1997; Rauch et al., 1996). In addition to hemispheric asymmetries, AP plane differences have also been found in our laboratory. For instance, Adamec et al. (2004) have shown that amygdala nuclei, depending on AP plane, produce different behavioral effects when kindled. As has been suggested for the effects of kindling on behavior (Adamec et al., 2004), present results indicate that it may be inappropriate to treat any anatomically defined nucleus of the amygdala as a functional unit when examining pCREB-lir. Similar to the amygdala, AP plane differences in CREB-lir have also been seen in the VTA. Olson et al. (2005) found that CREB activation within the rostral versus caudal sub-regions of the VTA produced opposite effects on drug reward. An important next

step to understanding hemispheric and AP plane-dependent alterations in pCREB-lir is to increase or decrease pCREB-lir (using techniques described above) in particular amygdala nuclei in a specific AP plane position (in a particular hemisphere) and assess its effect on ALB. From data described in Chapter 3, I suspect that increases in pCREB (using a PKA facilitator or CREB over-expression) in the anterior AP plane range in most amygdala nuclei (in both hemispheres), the BM, BLa, BLv and ACo (in the mid AP plane range), and the right lateral PAG, should produce long-lasting increases in ALB. This is hypothesized because pCREB-lir is elevated after predator stress and predator stress increases ALB. Furthermore, Wallace et al. (2004) have found increased ALB after CREB over-expression in the BLa. Areas where pCREB tended to be suppressed after predator stress should also be examined (i.e., especially the mid Ce) using a PKA inhibitor because Pandey et al. (2003) have found increases in ALB with reduced pCREB levels in this area. Moreover, according to Pandey et al. (2003) at least in the mid Ce, PKA facilitators should have no effect on ALB in normal rats. However, they have shown that a PKA facilitator infused into the mid-posterior Ce increased phosphorylation of CREB *and* prevented the development of anxiety in ethanol-withdrawn rats. Because of this, I predict that rats given a PKA facilitator in the mid-posterior Ce after predator stress should show an anxiolytic effect.

It is also of interest to identify potential effector genes that are activated by pCREB/CREB that may be influencing the behavioral changes after predator stress. In particular, peptides such as neuropeptide Y and cholecystokinin (CCK) are CREB-related target genes (Chance, Sheriff, Peng, & Balasubramaniam, 2000; Hansen & Nielsen,

2001; McClung, & Nestler, 2003; Pandey et al., 2004). Furthermore, both NPY and CCK have been localized within the amygdala (McDonald & Pearson, 1989) and have been implicated in mediating various aspects of stress, anxiety, and depression (Belcheva, Belcheva, Petkov, & Petkov, 1994; Heilig, 2004; Heilig, McLeod, Brot, Heinrichs, Menzaghi, Koob, Britton, 1993; Heilig & Widerlov 1990; Pandey, 2003). CCK is particularly interesting because CCK receptor block 30 min before, or after, cat exposure prevents increases in ALB assessed one week later in the EPM (Adamec et al., 1997). Studies that use RT-PCR for CCK mRNA and ICC for the CCK protein after predator stress may provide further insight into the mechanisms of change that occur in response to severe stress.

The second implication of our results is that predator stress-induced changes in pCREB-lir in particular brain areas may mediate increases in ALB following stress. This is because predator stress-induced changes in pCREB-lir in several amygdala nuclei, the right lPAG, and the BNST are NMDA receptor-dependent as are increases in ALB after predator stress (Adamec et al., 1999a; Adamec et al., 1999b; Blundell et al., 2005 - Chapter 2). This is consistent with the results of Wallace et al. (2004) in which CREB over-expression in the mid BLa produced increased ALB as measured in the EPM. If this hypothesis is correct, over-expression of CREB or facilitation of pCREB should increase ALB, at least in those brain areas that show an elevation in pCREB after predator stress, (i.e., most amygdala nuclei and the right lPAG). In contrast, pCREB manipulation via PKA inhibitors infused into the amygdala and/or lPAG before predator stress should block increases in ALB by preventing the NMDA receptor-dependent elevation in

phosphorylation of CREB in several amygdala nuclei and the right lPAG that occurs in response to predator stress. On the other hand, since pCREB-lir is suppressed in the BNST after predator stress, CREB over-expression should cause reduced ALB in predator stressed rats, assuming the BNST is involved in the anxiogenic effects of predator stress. On the other hand, PKA inhibitors infused into the BNST should increase ALB in control rats. As mentioned above, both acute and possible sustained effects of these manipulations should be investigated.

An unusual finding which requires further study is the elevation in pCREB-lir in the mid left BM, BLa, Ce, La, Me, ACo, and posterior BM, PCo, BLa, BLv, La, and left Me in predator stressed rats given CPP compared to predator stressed rats alone. It is unclear what this increase implies. However, areas where pCREB-lir is greater in the CPP group than the vehicle plus predator stress and predator stress alone groups, and predator stress is less than controls, may suggest an NMDA receptor-mediated tonic inhibition of pCREB (i.e., posterior BLa, PCo). Further studies of the effects on behavior of local injection of CPP into these brain areas are necessary to help clarify these results.

Third, predator stress-induced pCREB changes (at least in the lPAG) may mediate long-lasting LTP-like changes in amygdala afferent and efferent pathways following stress (Adamec et al., 2003; Chapter 3). Several lines of research suggest that pCREB mediates LTP-like changes in Ce-lPAG transmission, particularly the right Ce-lPAG pathway. First, pCREB-lir has been associated with long-lasting potentiation of neural transmission (Silva et al., 1998). Second, predator stress increases pCREB-lir in the right lPAG (Adamec et al., 2003). Third, predator stress-induced LTP-like changes in the Ce-

lPAG are longer lived in the right hemisphere (Adamec et al., 2003). Fourth, the same aspects of the stressor experience, and reaction to it, are predictive of both degree of pCREB-lir in the right lPAG and degree of potentiation in the right Ce-lPAG pathway (Adamec et al., 2003; Adamec et al., *in press*). Like predator stress-induced pCREB-lir, the longer-lived LTP-like changes in Ce-lPAG are also NMDA receptor-dependent and thus, present findings provide further support for the idea that long-lasting right Ce-lPAG LTP-like changes are pCREB-mediated. If this is the case, we hypothesize that enhanced pCREB levels (via PKA facilitator or CREB over-expression) in the right lPAG may increase Ce-lPAG neural transmission. Importantly, these changes may reflect neural circuit functional changes that mediate the behavioral changes (Adamec et al., 2001; Adamec et al., 2005a).

In the NMDA receptor-dependent VAB-BLa pathway, pCREB changes in the BLa are likely not involved in VAB-BLa potentiation because changes in pCREB in the BLa (in the AP plane position that corresponds to electrode placement) are not NMDA receptor-dependent. This conclusion is consistent with Maren and Fanselow's (1995) finding that NMDA dependent LTP in the VAB-BLa pathway may be presynaptic. Moreover, this result is somewhat consistent with Adamec et al. (2001) who showed a trend in paired pulse suppression evidence for presynaptic changes in VAB-BLa transmission nine days after predator stress. Thus, we hypothesize that altering pCREB levels in the posterior BLa (site of recording in the VAB-BLa pathways) should not alter potentiation in this pathway. Of course, future studies assessing the effects of pCREB

manipulation (in the amygdala and right lPAG) on neural transmission in both the Ce-lPAG and VAB-BLa pathways must be completed to test these hypotheses.

5.2.3 Changes in pCREB-lir After EPM in Predator Stressed Rats - Relationship to ALB and Amygdala Afferent and Efferent Transmission

As discussed in Chapter 4, exposure to the EPM alone did not produce changes in pCREB-lir in areas known to be involved in stress (Adamec et al., 2003; Adamec et al., *in press*). The lack of pCREB-lir changes after EPM exposure in naive rats suggests that a more severe stressor, such as predator stress, may be required to activate the pCREB system. Other stressors, such as submersion stress, which is currently being tested in our laboratory as well as others (Kavushansky, Vouimba, Cohen, Richter-Levin, *in press*), produce similar increases in ALB as predator stress and thus, may activate the pCREB system, presumably showing a comparable pattern of pCREB-lir as predator stress.

In addition, changes in pCREB-lir in the PAG were seen after the EPM in animals previously exposed to a cat. Importantly, these findings suggest mechanisms associated with neuroplasticity may be further engaged by relatively mild stresses in animals with a history of severe stress exposure. This is particularly important as a key feature of posttraumatic stress disorder (PTSD) is the exaggerated reaction to a mild stressor in which the response is more appropriate to the original traumatic situation than the current conditions (Bremner et al., 1995; Dykmanet al., 1997; Friedman, 1994).

As discussed in Chapter 4, one possible explanation for elevated pCREB-lir in the PAG after exposure to the EPM in predator stressed rats is as follows. pCREB-lir may be a "barometer" for state of responsiveness of the fear circuit. Responsiveness of a PAG NMDA receptor-dependent anxiety promoting system may be lastingly increased by LTP-like changes in the right Ce-lPAG pathway known to be produced by predator stress (Adamec et al., 2001; Adamec et al., 2005a). There are several lines of evidence that support this hypothesis. First, predator stress is anxiogenic in the EPM and potentiates Ce-lPAG neural transmission, both in an NMDA receptor-dependent manner (Adamec et al., 1999a; Adamec et al., 2005b). Second, Ce-lPAG transmission may in part, drive enhanced EPM anxiety (Adamec et al., 2005a). This suggests that exposure to the EPM activates the Ce-lPAG pathway and if the pathway is potentiated by prior predator stress, enhanced anxiety is observed. Under this hypothesis, in naive rats, EPM exposure produces little pCREB activation via glutamate/NMDA receptor activation because the NMDA/pCREB system is only slightly engaged. However, in rats with a history of predator stress and Ce-lPAG LTP-like changes, EPM activation of the NMDA/pCREB system is enhanced sufficiently to induce further pCREB-lir. These ideas require further experimentation to test their validity. If this model is correct, an NMDA receptor antagonist given prior to exposure to the EPM in predator stressed rats should block the anxiogenic response in the EPM and block the elevated pCREB-lir in the PAG. In addition, local inhibition of the Ce with lidocaine should have the same effect.

This model might also predict altered pCREB-lir in the Ce, which was not seen in the present study. One plausible explanation is that LTP-like changes in the Ce-lPAG

pathway appear to be post-synaptic (Adamec et al., 2001) which suggests that EPM induced NMDA receptor-dependent changes in pCREB should only occur in the PAG, and not the Ce.

In addition, increased pCREB-lir in the BLa after exposure to the EPM in predator stressed rats may also be expected for several reasons. First, predator stress-induced changes in ALB are blocked with microinjection of an NMDA receptor antagonist into the amygdala (Adamec et al., 1999b). Second, like the Ce-lPAG pathway, predator stress potentiates right VAB-BLa neural transmission in an NMDA receptor-dependent manner (Adamec et al., 2005b). Finally, VAB-BLa transmission also in part, drives enhanced EPM anxiety (Adamec et al., 2005a). These results suggest that exposure to the EPM activates the VAB-BLa pathway and if this pathway is potentiated by prior predator stress, enhanced anxiety is observed. Thus, in rats with a history of predator stress and VAB-BLa LTP-like changes, EPM activation of the NMDA/pCREB system should be enhanced sufficiently to induce changes in pCREB-lir in the BLa. As mentioned above, this was not the case, as pCREB-lir was not enhanced in the BLa. This finding may reflect the fact that NMDA receptor-dependent LTP-like changes in the VAB-BLa pathway may be pre-synaptic (Maren and Fanselow, 1995; Adamec et al. 2001). Again, further studies are needed to assess the behavioral effects of cutting the VAB and of CPP microinjected into posterior BLa prior to EPM exposure in predator stressed rats.

5.3 Densitometry Versus Stereological Cell Counting

As discussed in Chapter 3, predator stress increases density of pCREB lir in the right lPAG and amygdala, but does not increase the number of cells containing pCREB (Adamec et al., 2003; Adamec et al., *in press*; Chapter 3). Since previous studies suggest that predator stress may increase the amount of pCREB-lir within each cell, not the number of cells containing pCREB, we developed a modified stereological cell counting technique, which involved counting darkly stained pCREB cells separately from other pCREB stained cells. Results showed that total number of light and dark cells did not differ across groups. There was a trend for dark cells to appear in predator stressed rats, although only two of the six rats in this group displayed dark cells. This indicates that intermediate staining cells are promoting the densitometry differences and that intensity of moderate staining cells is higher in predator stressed rats than control or CPP groups. Because of this, we suggest that cell counting alone is not sufficient for detecting changes in pCREB in all cases. This is consistent with Swank (2000) who found that regions where pCREB was expressed (amygdala and cortex) after fear conditioning showed no increase in the number of immunoreactive nuclei, but showed an increase in the intensity of immunostaining. Further studies that measure intensity of pCREB within each cell, although time consuming, may be necessary to fully understand these results.

5.4 Conclusions

Results from this dissertation indicate that most changes in ALB following predator stress are NMDA receptor-dependent (Chapter 2). In addition, changes in

pCREB expression in several amygdala nuclei, the right lPAG and the BNST may mediate the predator stress-induced increases in ALB (Chapter 3). Furthermore, mechanisms associated with neuroplasticity may be engaged by relatively mild stresses in animals with a history of severe stress exposure (Chapter 4). To conclude, future investigation of the effects of CREB manipulation on ALB is warranted.

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Appendix 1

Description of Behavioral Tests

Hole Board: The hole board was a square wooden box (60 long cm x 60 cm wide x 35 cm high) and painted with grey enamel. There were four evenly spaced holes drilled in the floor of the box elevated 12 cm above the ground. The holes formed a square 14 cm from the walls of the box.

Elevated Plus Maze (EPM): The elevated plus maze consisted of four arms arranged in the shape of a plus sign. Each arm was 10 cm wide, 50 cm long and elevated 50 cm above the ground. The four arms were joined at the center by a 10 cm square platform. Two of the arms opposite each other had no sides while the other two arms had walls 40 cm high and were open at the top. The walls did not extend into the center of the maze. The maze was painted with flat grey enamel paint.

Light/Dark Box: The light/dark box was a single alley apparatus constructed of.5 in. plywood, divided into two chambers of equal size. Each chamber was 31.75 cm long, 10.48 cm wide and 14.6 cm high. Both chambers were covered by a transparent Plexiglass top hinged so it could be opened. Both tops had center pieces cut out to provide ventilation. One chamber had a solid wooden floor and was painted white. The other chamber had a metal mesh floor and its walls were painted black. The chamber painted black had its Plexiglass top rendered opaque with a black plastic covering. In addition, a 100 W lamp was positioned 66 cm above the white chamber. Finally testing

took place in a darkened room illuminated only by the lamp over the white chamber. This produced a light intensity at the center of the floor of the white chamber of 55 fc, and an intensity of 2 fc at the center of the floor of the dark chamber. Behavior in the testing apparatus was videotaped for later analysis with a video camera mounted directly over the apparatus.

Haller Box: The Haller Box was a three chamber box with outside dimensions of 66 cm long x 42.5 cm wide x 30.5 cm high. The rat being tested was initially placed in the start chamber, separated from a large middle chamber by a rounded entrance measuring 10.2 cm in diameter closed off by a clear plastic guillotine door. The middle chamber included a far chamber separated by a clear Plexiglas wall with holes in it. A stimulus rat was placed in this far chamber. The dimensions of the start, middle and far chambers were: 21 cm x 42.5 cm x 30.5 cm high; 24.8 cm x 42.5 cm x 30.5 cm high; and 15.2 cm x 42.5 cm x 30.5 cm high, respectively.

Appendix 2

Immunocytochemistry pCREB Protocol

1. Wash sections 3 times for 10 minutes each (can be left up to 45 minutes) with phosphate buffered saline (PBS). Approximately 1 ml of PBS per well.

2. Blot with a kimwipe

3. 1 ml of solution per well (solution = 10% normal goat serum (NGS) + 0.1% Triton X-100 in PBS). Cover with parafilm and place on rocker for 1 hour.

4. Wash 3 times for 10 minutes each with PBS.

5. Blot as in step 2

6. Have primary anitbody diluted and ready to use. Dilute primary antibody (rabbit anti-rat) in a solution of PBS, containing 2% NGS and 0.1% Triton X-100 (swirl, do not shake).

7. 1 ml of solution (step 6) into each well and incubate for 24 or 48 hrs (re-used primary antibody). Cover wells with parafilm to prevent drying.

8. Wash 3 times for 10 minutes each with PBS

9. Blot as in step 2

10. Prepare secondary biotinylated antibody (goat anti-rabbit). Use the same diluent as for the primary antibody. For 10 ml of buffer, use 50 µl of secondary antibody. Add 1 ml of solution to each well. Cover with parafilm and agitate (on rocker) for 1 hour.

11. At this time, prepare the avidin-biotin complex (ABC) reagent as the Vector Stain kit instructs. To 5 ml of PBS, add 50 µl of reagent A and mix. Then add 50 µl of reagent B, mix well.

* This solution must be made at least 30 minutes prior to use.

12. Wash 3 times for 10 minutes each with PBS.

13. Blot as in step 2.

14. Add 1 ml of ABC solution to each well and incubate for 1 hour on rocker.

15. Wash 3 times for 10 minutes each with PBS.

16. Blot as in step 2.

17. Make diaminobenzadine (DAB) solution just prior to use - use gloves

    (a) Add 10 mg tablet to 10 ml of PBS, vortex until tablet has dissolved

    (b) While swirling, add another 10 ml of PBS to DAB solution

    (c) Just prior to adding DAB solution to sections, add 60 µl of H_2O_2 (0.1 ml of 30% H_2O_2 in 0.9 ml of PBS)

18. Add at least 1 ml to each well. Incubate (on agitator) for 5-25 minutes, monitoring for staining.

19. Remove wells from DAB solution.

20. Blot as in step 2.

21. Wash 3 times for 10 minutes in PBS.

20. Leave in PBS overnight and cover with parafilm.

21. Mount sections on slides.

22. Dehydration series:

    1. distilled H20

    2. 50% ethanol

    3. 75% ethanol

4. 90% ethanol

5. 90% ethanol

6. 100% ethanol

7. 100% ethanol

8. Xylene

9. Xylene

23. Coverslip.