# INVESTIGATION OF MECHANISMS AND LOCUS OF NEURAL CHANGE UNDERLYING STRESS-INDUCED CHANGES IN AFFECT

CENTRE FOR NEWFOUNDLAND STUDIES

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# Investigation of Mechanisms and Locus of Neural Change Underlying Stress-Induced Changes in Affect

by

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A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Psychology

Memorial University of Newfoundland

2001

St. John's Newfoundland

# Abstract

Stress has been linked to a variety of affective disorders, in particular, posttraumatic stress disorder (PTSD). PTSD is a long-lasting, chronic psychological disorder that results from exposure to a life threatening, inescapable stressor. There is no known cure or adequate treatment for this disease. In addition, there is little information regarding its cause. Brain imaging studies have implicated hyperexcitability of the right amygdala in PTSD and in response to traumatic events. Recent animal research has confirmed the role of the amygdala during severe stress. The goal of this study was to identify particular neural pathways and structures in which changes in function mediate particular changes in affect following predator stress. In this set of experiments, predator stress was achieved by exposing a rat to a cat for five minutes.

Neural transmission of amygdala afferent and efferent pathways was examined in Experiment 1. Specifically, pathways studied were the ventral angular bundle input to the basolateral amygdala and central and basolateral amygdala output to the periaqueductal gray (PAG). Transmission was investigated one day (Day 1 study) and nine days (Day 9 study) after predator stress. Behavioral testing occurred seven days post predator stress in the Day 9 study. Predator stress was "anxiogenic" in the elevated plus maze, light/dark box and acoustic startle tests.

Lasting changes were also observed in neural transmission in both the Day 1 and Day 9 studies. Predator stress appeared to potentiate right and depotentiate left hemisphere afferent amygdala transmission. In contrast, predator stress potentiated amygdala efferent transmission to both right and left PAG, depending on the amygdala

nucleus stimulated. Paired pulse and intensity series analyses suggest that transmission changes may be post synaptic or presynaptic, depending on the pathway. These results are similar to results found in cat studies, but there are species differences.

PCREB expression in the amygdala, PAG and ventral medial hypothalamus was examined in Experiment 2. Three groups were used in this experiment: Predator stressed (same procedure as used in electrophysiology study), Handled control and Restrained control. The restrained group was added to mimic the periods of immobility of cat exposed rats.

Predator stress increased pCREB expression in the right lateral column of the PAG and a subset of nuclei in the amygdala. In addition, restraining the rat reduced pCREB expression in the left lateral and ventral columns of the PAG. There were no differences in pCREB intensity in the ventral angular bundle.

Both experiments confirm the role of the amydala and PAG in predator stress-induced changes in affective behavior. In addition, as seen in cat and human data, these experiments appear to support the importance of the right hemisphere in stress.

# Acknowledgements

I would like to thank Dr. Robert Adamec, my thesis supervisor, for his patience, support, guidance, and humor while I completed my thesis. His depth of knowledge and love for research is unparalleled. Sincere gratitude goes to Dr. Charles Malsbury for his support and encouragement. As well, I would like to thank Dr. Carolyn Harley and Dr. Charles Malsbury for acting as members of my supervisory committee.

I would like to thank Andrew Collins for making the long Fridays in the lab enjoyable. Andrew, along with Paul Burton, must also be thanked for behavioral data collection.

A very special thank-you goes to my family, without their encouragement and support none of this would have been possible.

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# Investigation of Mechanisms and Locus of Neural Change Underlying Stress-Induced Changes in Affect.

The goal of this study is to investigate the neural basis of lasting changes in affect produced by severe stress. Anxiety often occurs in humans after exposure to a traumatic event. Normal fear and anxious apprehension are emotional responses to danger or threat and motivate the individual to relieve the negative emotional state. When an animal is faced with danger, threat or an aversive situation, an adaptive fear response is elicited and then subsides when the aversion diminishes. This differs from pathological anxiety disorders in which the emotional responses are chronically dysfunctional.

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 R.C., Sonnega A., Bromet E., Hughes M., & Nelson C.B, 1995). More importantly, of those percentages, 15 % may develop post traumatic stress disorder (PTSD) (Kessler *et al*, 1995). PTSD can be a debilitating disorder characterized by severe anxiety, nightmares, agitation and often depression. The traumatic event is persistently reexperienced 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. There is no cure for this disorder; thus the study of the effects of stress on brain physiology is particularly important.

I will begin with an examination of the symptoms and criteria associated with PTSD. The various animal models used to understand PTSD will be described including the model used in this study. I will conclude with a discussion of relevant research on both felines and rodents which will lead into the justification for this study.

There are six criteria outlined in the DSM IV-R and of those, at least two must be present for a diagnosis of PTSD. 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. In addition, if the symptoms have been 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.

In addition to the diagnostic criteria for PTSD, biochemical abnormalities have been documented in patients with PTSD. PTSD patients have abnormal stress hormone release (Van der Kolk, 1994). Specifically, abnormal levels of modulators such as norepinephrine, oxytocin, cortisol and vasopressin have been found in the blood of PTSD patients. It has also been suggested that persistent alteration in stress hormone secretion alters memory processing in these patients (Van der Kolk, 1994).

Much research has been done and is currently underway on patients with PTSD. Perhaps the most widely explored test is the startle response. Butler, Braff, Rausch, Jenkins, Sprock, and Geyer (1990) tested Vietnam veterans with PTSD and

non-PTSD veterans for acoustic response using eyeblink electromyogram amplitudes. PTSD patients exhibited higher eyeblink amplitudes than the non-PTSD veterans. In addition, a study by Kolb (1987) found differences in PTSD and controls in both blood pressure and galvanic skin response when presented with combat sounds. PTSD veterans showed a greater response to the sounds than the controls.

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

## Models of PTSD

Animal models are very useful because they allow the opportunity to simulate a human condition in a controlled setting; the disease 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, and affect the startle response both by enhancing it and delaying its habituation. The following section will examine four models used to understand PTSD

*Classical Conditioning Model*

Classical conditioning links the trauma with the symptoms of PTSD. It has been suggested that the feeling 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 (Kolb & Mulyipassi, 1982). In theory this appears to be a good explanation, however, it has been argued that conditioning does not account for the finding of the exaggerated startle response nor can it explain patients with delay of onset PTSD (Pitman, Orr & Shalev, 1993; Shalev, 1993).

*Inescapable Shock Model*

Van der Kolk, Greenberg, Boyd, and Krystal (1985) suggest inescapable shock (IS) as a model for PTSD. Animals that have experienced IS later exhibit decreased initiation of behavior, cognitive deficits and symptoms of emotional disruption (Rosen & Fields, 1988).

Biochemical results support this model for PTSD. For example, exposure to IS increases NE turnover, increases plasma catecholamine levels, depletes central NE and increases MHPG production (Van der Kolk et al., 1985). This model, however, has been criticized for several reasons. The model fails to adequately explain the chronicity and delayed development of some of the symptoms of PTSD (Yehuda & Antelman, 1993). In addition, Yehuda and Antelman (1993) state that the IS model does not account for the possibility of developing PTSD after a single, brief exposure to trauma. Finally, the model does not take into consideration the effect of stressor intensity which appears to be relevant to the severity of PTSD (Yehuda et al., 1993).

*Kindling Model*

Kindling refers to a process by which repeated presentation of subthreshold stimuli, such as electrical or chemical stimulation, sensitizes limbic circuits and leads to lowered thresholds for electrically elicited seizures (Martin, 1991). Nieminen, Sirvio, Teittinen, Pitkanen, Airaksinen, and Riekkinen (1992) have shown that kindling of the basolateral amygdala in rats increases anxiety in the elevated plus maze. Studies in the Adamec laboratory have confirmed that finding (Adamec, 1990; Adamec & McKay, 1993). More detail will be given further in the paper regarding the effects of kindling on limbic structures. Criticism of this model, by Pitmann and colleagues (1993), is based on the fact that kindling has an electrophysiological basis, as opposed to a behavioral basis.

*Emotive Biasing Model*

The final model of PTSD examined is emotive biasing (Adamec, 1978). Pitman, Orr and Shalev (1993) have suggested that emotive biasing may account for the lasting changes in emotional disposition found in PTSD. This model involves sensitization and kindling. Specifically, repeated stimulation of a limbic substrate which is associated with a certain emotional state eventually alters the substrate, enhancing its function (Adamec, 1978). This idea is consistent with Kolb's (1987) theory which states that PTSD is the result of cortical neuronal death and synaptic change. It occurs as a result of excessive and prolonged sensitization of limbic structures in response to trauma. This is particularly relevant as PTSD patients suffer

from generalized heightened arousal as well as physiological reactions to trauma related stimuli in the environment.

The next section will review some of the relevant research on the effects of severe stress on felines and rodents using both pharmacological and electrophysiological techniques.

## Brain Areas Involved in Anxiety

In this section, the regions of the brain which are involved in fear and anxiety will be explored. All three areas of research implicate the amygdala and the periacqueductal gray (PAG) in fear and anxiety.

Patients with selective bilateral lesions of the amygdala lack the ability to recognize specific facial and vocal expressions of fear (Adolphs, Tranel, Damasio, & Damasio, 1994). Moreover, patients with selective damage to the amygdala do not display emotional responses (e.g. increases skin conductance) to conditioned aversive stimuli (LaBar, LeDoux, Spencer, & Phelps, 1995). 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, 1997). This hemispheric difference was also seen in a study by Rauch and colleagues. Using a PET scan, they 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, and Pitman, 1995). Data in

both felines and rodents suggest a similar hemispheric specialization which will be examined in the following sections.

In addition to the amygdala, the periacqueductal gray (PAG) is responsible for fear-related freezing and escape/defensive behaviors in animals (fight or flight response) (Adamec, Kent, Anisman, Shallow & Merali, 1998). There are parallels in humans. Nashold, Wilson, & Slaughter (1969) reported that stimulation of the PAG in human patients caused feelings of "fearfulness" and "frightfulness". Patients became apprehensive and would not allow further stimulation of the area.

## Behavioral and Physiological Research 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. These drugs bind to the benzodiazepine receptor (BZR). There are various types of benzodiazepine drugs available which include agonists, inverse agonists and antagonists. Benzodiazepine agonists tend to be anxiolytic, such as Valium. Some inverse benzodiazepine agonists, (*e.g.* N-methyl-beta carboline, 3 carboximide or FG), cause intense anxiety in humans and 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).

*Behavioral Research in Felines*

Effects of FG are blocked by anxiolytics in humans (Doyer, Errington, Laroche, & Bliss, 1996) and BZR antagonists, such as Flumazenil, in animals (Ongini, 1983). Using FG, Adamec showed that this pharmacological stress produces 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 (Adamec, 1991). The behaviors measured are considered to be an index of cat anxiety.

*Physiological Research in Felines*

FG produces equally long lasting changes in limbic physiology, which closely correlate with behavioral changes in cats. Evoked potential methods were used to assess changes in limbic pathways known to be involved in the control of feline defensiveness. It has been found that long term potentiation (LTP) in neural transmission from right amygdala to right lateral column of the periacqueductal gray of the cat is critical for lasting changes in affect produced by pharmacological stress (FG) (Adamec, 1997; Adamec, 1998; Adamec, 1998). LTP can be described as long-lasting enhancements in synaptic efficacy that have the properties expected of a memory mechanism (e.g. long-lasting, associativity, and reversibility) (Izquieredo, 1994). LTP is not a single phenomenon; rather there are various forms of LTP with distinct time courses and distinct mechanisms. It is known that some, but not all types of LTP depend on the activation of NMDA receptors (Adamec, 1997; Adamec, 1998, Adamec, Kent, Anisman, Shallow & Merali, 1998; Adamec, Burton, Shallow

& Budgell, 1999; Maren, 1996; Rogen & LeDoux, 1995). The NMDA receptor is a type of glutamate receptor, linked to a calcium channel and causes excitation in the cell. Calcium influx also triggers enzymatic cascades which lead to lasting changes in synaptic transmission (Massicotte, 2000).

It has been found that the NMDA receptor blocker, AP7, administered before FG prevented both long lasting increases in excitatory transmission in amygdala efferents and the behavioral response to FG in cats (Adamec, 1998; Adamec, 1998). Therefore an NMDA dependent form of LTP in amygdala efferents may underlie the behavioral changes produced by FG. Similar effects on brain and behavior may be produced by amygdala sensitization following focal seizures (partial kindling). Moreover, depotentiation of LTP in amygdala efferents with low frequency stimulation protocols reverses both LTP and the behavioral effects of partial kindling (Adamec, 1999). The changes in LTP in the right amygdalo-PAG pathway are specifically related to changes in defensive response but not to changes in predatory behavior, as selective depotentiation of this pathway reverses effects of partial kindling on defensive behavior, but not on predatory behavior (Adamec, 1999). A similar relationship between defensive behavior and right amygdalo-PAG LTP has been seen in the FG model in cats (Adamec, 1997). Together, these data suggest that multiple system changes may mediate multiple behavioral changes following stress or epileptic sensitization. Findings in rodents are similar and will be discussed in the next section. First, however, the model used in this study to create a severely stressful event will be examined.

## Model of PTSD in Current Study

The model used in this study was developed in the Adamec laboratory (Adamec & Shallow, 1993). It involves unprotected exposure of a rat to a cat for five minutes. The rat is placed in a room with a cat so that the cat can approach and gently paw the rat. Exposure to a cat produces a long-lasting increase in rat anxiety-like behavior lasting at least three weeks after the exposure (Adamec, 1997; Adamec & Shallow, 1993, Adamec, Shallow & Budgell, 1997). Behaviors examined include response to a novel hole board, plus maze, and unconditioned acoustic startle. The model has a high degree of face validity due to the nature of the stressor.

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, the animal would have experienced chronic anxiety for at least 18 months of a human's life span. This time line meets the criterion as set out by the DSM IV-R where anxiety is considered chronic if it persists for three months or longer.

This model is relevant, as brain areas implicated in behavioral changes in animals as a result of predator stress resemble those areas which are thought to mediate aspects of the symptoms of PTSD. For example, parallel path analytic studies have been done using data from Vietnam veterans and rodents to determine if 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, the higher the level of anxiety in the rat. Also, individual differences play a role in the

response to stress both in rats and humans (Pitman, 1997). Finally, similar lasting changes in startability and habituation of startle are seen in both rats and humans (Adamec, Kent, Anisman, Shallow & Merali, 1998).

## Behavioral and Physiological Research in Rodents

Much of the rodent work is an extension of cat research. As discussed earlier, NMDA dependent changes in particular neural pathways appear to alter particular behaviors. This has been documented in the cat, as different pathways appear to be involved in defensive and predatory behaviors. Now similar evidence exists in the rat.

As in the cat, initiation, but not maintenance, of increases in rat anxiety and startle are dependent on NMDA receptors (Adamec, Burton, Shallow & Budgell, 1999; Adamec, Burton, Shallow & Budgell, 1999). Increases in rat anxiety are prevented by systemic injection of NMDA blockers before, but not after, cat exposure (Adamec, Burton, Shallow & Budgell, 1999). Moreover, a local NMDA receptor block may achieve selective blockade of particular behavioral changes in the amygdala of rats (Adamec et al., 1999). Local injection of the NMDA antagonist MK-801, in the right dorsal amygdala 30 minutes prior to cat exposure prevented the expected increase in acoustic startle measured one week later. Right amygdala injections of MK-801, however, did not prevent decreased open arm exploration and decreased risk assessment in the elevated plus maze. 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 plus maze measured one week later, while leaving intact decreases in open arm exploration and increases in acoustic startle response. Taken together, the cat and rat findings are consistent with the view that NMDA dependent LTP in particular amygdala circuits may underlie particular behavioral changes in response to stress.

Behavioral effects of limbic sensitization produced by kindling also show hemispheric asymmetries. Adamec and Morgan (1994) have shown that kindling of the left medial/basolateral amygdala decreases plus maze anxiety (anxiolytic) while kindling of the right hemisphere in analogous nuclei increases plus maze anxiety (anxiogenic).

In addition to hemispheric differences, the degree of plus maze anxiety following kindling was also dependent on kindling electrode placement in the anterior-posterior plane. The relationship between position in the AP plane and behavioral effect of kindling was dependent on the nucleus being kindled (Adamec & Morgan, 1994). For example, increases in anxiety 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 plus maze.

Other research implicates the right amygdala in fear and anxiety. Coleman-Mesches and McGaugh (1995) have demonstrated that amygdala in the left and right hemispheres play different roles in the acquisition and expression of fear. Cannulas

were placed either bilaterally or unilaterally in the amygdala. Animals were given either an infusion of lidocaine hydrochloride or a neutral buffer five minutes before training on an inhibitory avoidance task. Retention was tested two days post infusion. Some of the animals were retrained at this time and tested again two days later. Animals given bilateral infusions of lidocaine prior to the initial training were impaired on acquisition, retention and subsequently the relearning of the task at a later time. Unilateral infusions of lidocaine did not affect acquisition yet rats given lidocaine in the right amygdala were impaired on retention two days later.

As seen in the cat, the rodent data imply that particular amygdala circuits in a particular hemisphere may underlie particular behavioral changes in response to stress.

*Anxiety, PAG and Rodents*

In addition to the amygdala, the PAG is also an important part of the neural substrate of anxiety. Activation of the PAG has been implicated in anxiety-like behavior (ALB) (Brandao, Anseloni, Pandossio, De Araujo & Castilho, 1999). Manipulation of a variety of receptor systems (NMDA, GABA, noradrenergic, benzodiazepine, substance P, opioid, corticotrophin releasing factor) in the PAG alters ALB in the plus maze and social interaction tests (Adamec, 1999). Microinjection of the benzodiazepine agonist, midazolam (80 nMol) into the dorsal PAG dose-dependently decreases anxiety in the elevated plus maze. (Russo et al, 1993). NMDA receptors in the PAG also appear to be involved in anxiety. Local block of NMDA receptors in the PAG, with the competitive antagonist AP7, dose

dependently decreases plus maze anxiety (Guimaraes, Carobrez, De Aguiar, and Graeff, 1991).

## Justification for Present Study

Both the cat and rat data suggest the importance of the right amygdalo-PAG pathway in anxiety. The cat research implies that LTP in particular pathways affects particular behaviors. The rodent data also support this conclusion. This study was designed to further investigate the relationships between neural pathways and behavior. In view of the evidence for hemispheric asymmetries, pathways will be examined electrophysiologically for hemispheric differences. The neural pathways chosen reflect pathways relevant in fear and anxiety. Some of the pathways examined use glutamatergic receptors and support glutamate dependent LTP associated with the conditioning of defensive responses.

Three neural pathways were probed in both hemispheres electrophysiologically using evoked potential methods. The afferent amygdala pathway investigated was the monosynaptic input to the basolateral amygdala (BLA) from the ventral angular bundle (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). Infusion of an NMDA receptor antagonist (APV) into the BLA before training disrupted the acquisition of

contextual fear. Fear conditioning was not affected, however, by a posttraining APV infusion into the BLA (Maren, Aharonov & Fanselow, 1996).

The efferent pathways examined were the central nucleus (ACe) to the PAG (ACe-PAG) and BLA to the PAG (BLA-PAG). The central nucleus has been implicated in rodent defensiveness and anxiety-like behaviors (Davis, 1992). Moreover, the ACe is activated in stress-induced increases in anxiety-like behavior (Shepard, Barron & Myers, 2000). Shepard *et al.* (2000) examined the effects of the delivery of corticosterone to the ACe on anxiety-like behavior (ALB). Corticosterone implants increased indices of ALB on the elevated-plus maze which support the involvement of the amygdala in anxiety. The pathway between the ACe and PAG has also been studied. Carrive, Lee and Su have shown the pathway to be involved in contextually conditioned fear (1999). They looked at changes in FOS expression after a unilateral blockade of the central amygdalofugal pathway with lidocaine. This pathway contains fibers originating from the ACe that project directly to the PAG. The results show that when lidocaine was injected into the ACe or the amygdalofugal pathway of conditioned rats, fear-induced FOS expression in the PAG was reduced. The PAG has been shown to be involved in rodent anxiety-like behavior (Brandao *et al.*, 1999) and is activated by predator stress (Canteras & Goto, 1999). Canteras and colleagues (1999) exposed a rat to a predator (cat) and examined the FOS expression in the PAG. After the predatory encounter, FOS-immunoreactive cells were observed in the dorsomedial, and dorsolateral areas of the PAG. In addition, microinjection of the benzodiazepine agonist, midazolam, into the dorsal PAG dose-dependently decreases anxiety in the elevated plus maze (Russo, Guimaraes, De Aguiar, and

Graeff, 1993). NMDA receptors in the PAG are also involved in rodent anxiety. Local block of the NMDA receptors in the PAG with AP7 dose dependently decreases plus maze anxiety (Guimaraes et al, 1991)

The second efferent pathway examined was the BLA-PAG. This pathway has also been studied electrophysiologically, and behavioral data implicate it in anxiety. For example, the BLA is involved in contextual fear conditioning (Maren et al, 1996), modulation of the startle response (Maren et at, 1996), and the ulceration response to stress (Ray & Henke, 1991). Mindy, Miserendino, Sananes, Melia, and Davis (1990) and Campeau, Miserendino and Davis (1992) used the startle paradigm as a model for anxiety. Infusion of the competitive NMDA antagonist AP5 bilaterally into the BLA blocked the acquisition of fear-potentiated startle. In addition, activation of the BLA excites dorsolateral and lateral PAG cells (Gomez, Chandler, & Behbehani, 1996). Finally, glutamatergic neural transmission is involved in BLA modulation of anxiety-like behavior (Sajdyk & Shekhar, 1997).

The present experiment compared neural transmission in the VAB-BLA and both the BLA-PAG and ACe-PAG pathways in a Predator stressed and a Control group. The measures were taken both one day and nine days post cat exposure to determine whether the effects of predator stress were long lasting. In addition, the longer interval permitted behavior testing with the intention of relating brain changes to behavior. Unfortunately, only day one or day nine data are available for some of the pathways. We hypothesized that predator stress would induce lasting potentiation of neural transmission in the right hemisphere in some or all of the pathways.

## *PCREB & predator stress*

As discussed above, NMDA-mediated neural plasticity contributes to lasting increases in rat anxiety produced by predator stress. To further identify the neural circuitry mediating behavioral changes due to stress, the induction of phosphorylated cyclic AMP response element binding protein (pCREB) was investigated. Shors and Servatius (1995) investigated the importance of glutamate and NMDA receptors. They determined that footshock-induced sensitization and facilitation of a fear-conditioned response require NMDA activation. PCREB is a marker of neural activity (Deisseroth, Bito, & Tsien, 1996). More importantly, pCREB is linked to gene transcriptional changes involved in maintenance of LTP and storage of long term memories in vertebrates (Silva, Kogen, Frankland, & Kida, 1998). Moreover, phosphorylation of CREB is regulated by NMDA receptors (Segal & Murphy, 1998). Therefore visualization of pCREB may be helpful in identifying brain structures in which NMDA-dependent LTP-like mechanisms mediate behavioral changes.

This study investigated pCREB expression in the PAG, amygdala, and ventral medial hypothalamus (VMH). Three groups of rats were used. Predator stress rats were exposed to a cat (as described above). The control group was handled only, while the restrained group was placed in a small container. The restrained control group was included to recreate the same periods of immobility as seen in cat-exposed rats. We hypothesized that predator stressed rats would express more pCREB in the PAG and amygdala than either the control or restrained groups. In addition, the right hemisphere of the PAG and particular nuclei in the amygdala should show more pCREB staining than the same regions in the left hemisphere.

In summary, the theory being tested suggests that the amygdala and its connections to the PAG play a central role in both normal fear and pathological anxiety (PTSD) (Adamec, 1997; Rosen & Schulkin, 1998). Hyperexcitability occurs by a process of neural sensitization in which a psychosocial stressor (cat) can initiate changes in the brain's fear circuits, which include amygdala and PAG, that lead to enhanced perception of and response to, subsequent threat and danger. Hyperexcitability in these circuits develops from an increase in a cascade of biomolecular events that include gene expression (pCREB). It is the degree of potentiation of the pathways and the biomolecular events in the amygdala and PAG induced by predator stress that are examined in this thesis project.

# Method

## PCREB

### *Animals*

Forty-five male hooded Long Evans rats (Rattus norvegicus) from the vivarium, MUN, were used in experiment one. 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 seven a.m. Rats weighed approximately 200g on arrival and between 230 and 280g on the day 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 rats attempted to escape and the grip was released as soon as the animal became still.

*Groups*

The rats were randomly assigned to one of three groups (n=15 animals per group). The three groups are handled control (HC), handled restrained (HR), and predator stressed (handled exposed). Until the day of testing, all rats were treated the same excluding the HR group. Rats in the HR group were placed in a restraint box for 2, 3, and 4 minutes a day on each of the three days prior to testing to habituate the rat to the restraint apparatus. The restraint box measured 17.5 cm long, 9.5 cm wide and 9 cm high. This was done after all rats were handled. After handling, all rats were then transported into a different room which contained the restraint apparatus. The restrained group was placed in the restraint box one at a time while all other rats were left undisturbed on the metal rack used to transport the animals. The restraint group was used to control for periods of immobility experienced by the rat which is often observed during exposure to the cat. The restraint box was a clear plastic container with air holes and a lid. The rat was placed inside the container and the lid closed. The container was then placed inside a sound proof box which was part of the startle apparatus. Movement of the rat was limited but not completely prevented. The sound proof box had two holes to allow the experimenter to observe the behavior of the rat. 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.

*Testing*

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

*Predator Stressed (Handled Exposed)*

The predator stressed group was exposed to a cat on the day of testing. Cat exposures were preformed in a large wooden room with carpet on the floor. For more details on the room, see Adamec (1980). The same cat was used for all exposed animals. Also, the cat was placed in the room at least one hour prior to testing. The rat was placed in a wooden enclosure and transported to the exposure room. The wooden enclosure measured 18.5 cm high, 19 cm long and 14.5 cm wide. The rat enclosure fit a small opening at the floor of the exposure room. This small door was opened and the rat 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 using two video cameras to ensure that all areas of the room could be visualized. The videotapes were manually switched back and forth depending on the best position to capture both the activities of 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 the rats but did not injure them. Rats were examined for wounds after the cat encounters, and none were observed. The rat was removed from the exposure room again without handling. This was accomplished using a soft broom to guide the rat back into the small enclosure. The cat remained in the room while the rat was removed. The enclosure door was closed and the animal was returned to its home cage. During the rat exposure, the home cage of the rat being tested was moved to a small dark room. After testing, the rat was placed back in its home cage and left undisturbed for ten minutes. Only the exposed rats were placed in this room. At this time, the rat received an overdose of sodium pentobarbital. Ten minutes later the rat was checked for reaction (if the rat still displayed a reflex, it was given a supplementary dose of 0.1 mL). If no reaction, the rat was perfused with 200 mL of heparinized saline and 500 mL of paraformaldehyde. The timing of the perfusion was important because it has been shown that expression of pCREB peaks between 20 and 25 minutes after exposure (Silva *et al.*, 1998). The brain was removed, placed in a 20% sucrose solution overnight and subsequently frozen in isopentane cooled by liquid nitrogen. The brain was left in a minus 70°C freezer until sectioning.

*Handled Control*

Rats in this group 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 handled for one minute. After handling, the rat was placed back in its home cage for ten minutes. Both the restrained and handled rats were placed in the

same darkened room which differed from the room where the predator stressed rats were kept. At this time, all groups were treated the same. The rat received an injection and ten minutes later, the rat was checked for a response. If there was no reaction, the rat was perfused, the brain removed, left to sink in sucrose and subsequently frozen in isopentane.

*Handled Restraint*

Similar to the handled control group, rats in this group did not come into contact with a cat, cat odors or a rat that had previously been exposed to a cat. This group followed similar procedures as the handled control and predator stressed groups, excluding the testing phase. The rats were weighed and then placed into the restraint box for five minutes. During the five-minute period, the amount of time the rat spent immobile was recorded. At this point the procedure was the same for all the groups. The rat was then placed back in its home cage for ten minutes. The rat received an injection and was left alone for ten minutes. The rat was checked for a response, and if unresponsive, the rat was perfused as described previously. The brain was removed, left to sink in sucrose overnight and then frozen.

*Immunocytochemistry*

Forty μm coronal sections were cut in a cryostat. All sections were cut by the same person using the same cryostat. Sections were taken from –5.8 to –6.8 mm behind bregma to capture the PAG and –2.3 to –3.3 mm behind bregma to capture the amygdala and VMH (Paxinos & Watson, 1982). These ranges were chosen because

they include the recording sites in the electrophysiology experiments. Every second section was saved which provided 24 sections from each brain for processing. A multiple of three brains, one brain from each group, was cut and processed at one time. Many procedures were tried to maximize staining. Sections processed using 1/500 dilution both for 24 and 48 hours (reused 1° antibody) were analyzed. In addition, brains were processed using three sections per well (one section from each condition: handled control, handled restrained, & predator stressed). The exact protocol used for staining can be found in Appendix 1.

Stained sections were mounted and later analyzed using image analysis software (Jandel, MOKA software). Densitometry (in calibrated optical density units) relative to the entire section (for PAG) or relative to an area of the internal capsule (for amygdala and VMH) was used to quantify the data. Hemispheres were measured separately. Examples of pCREB staining in the PAG appear in **Figure 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 quadrants were dorsal, the middle quadrants were lateral and the bottom quadrants were ventral.

The amygdala was also divided into its nuclei: central nucleus (ACe), basolateral nucleus (BLA), lateral nucleus (LA), basomedial nucleus (BMA), medial nucleus (MeAV) and cortical nucleus (ACo). The sections were divided into three

templates to define the nuclei. The nuclei were defined with coordinates in the rat atlas (Paxinos & Watson, 1982) and the exact coordinates, and templates used, can be found in Appendix 2. Coordinates set by the templates were mapped onto the actual section for densitometry analyses. Templates were designed to correct for tissue shrinkage. Again, right and left hemispheres were measured and analyzed separately.

Densitometry measures of the ventral medial hypothalamus were also taken in both the right and left hemispheres.

All densitometry measures were analyzed with ANOVAs examining Group and Hemisphere factors and Columnar factor in the PAG. Planned comparisons were done using t tests.

## Electrophysiology

The purpose of this study was to examine the effects of predator stress on neural transmission. This was achieved using two sets of experiments; Day 1 study examined neural transmission one-day post predator stress and the second experiment, Day 9 study, examined neural transmission nine days after predator stress.

*Subjects*

A total of 68 male hooded Long Evans (Rattus norvegicus) rats weighing 200g (50-52 days old) at the time of arrival were used in both studies. All rats were allowed one day acclimatization to the lab and their home cages. All rats were

housed singly in clear polycarbonate cages measuring 46 cm long x 24 cm wide x 20 cm deep and wood chip bedding covered the floor of the cages. Food and water were available ad lib and a 12 hour light cycle was used with lights on at 7 am. Rats were handled for three days as described above (pCREB study). Rats were randomly assigned to either Handled Control (HC) or Predator Stressed (handled exposed) groups.

*Cat Exposure*

The cat was placed into the room at least one hour prior to testing. The procedure used was the same as used above in the pCREB experiment for predator stress. The rat entered the cat room and was left unprotected with the cat for five minutes. The rat was returned to its home cage and was left untouched for either one day (Day 1 study) or one week (Day 9 study).

*Control*

Rats in the Handled Control group were handled for one minute on the day of cat exposure then returned to their home cages for either one day (Day 1 study) or one week (Day 9 study).

*Day 1 Study*

Rats were prepared for electrophysiological recording one day after predator stress or handling. There were 48 rats in this group: 24 handled controls and 24 predator stressed. These groups were further divided into 12 control and 12 predator

stressed in which the VAB-BLA transmission was investigated. The remaining 12 controls and 12 predator stressed were used to examine the ACe –PAG pathway.

*Day 9 Study*

There were 10 predator stressed and 10 controls in this group. The rats were subjected to behavioral testing seven days post exposure or handling. The tests used were the hole board, elevated plus maze, light/dark box and acoustic startle. Response to the hole board and elevated plus maze was assessed on one day and response to acoustic startle and light/dark box was assessed on another day. Order of testing of behavioral response was counterbalanced across both rat and group. On day 9 after cat exposure (or handling) and one day post behavioral testing, rats were prepared for electrophysiological recording. In these rats, both the VAB- BLA and the BLA-PAG were explored, one after another in that order to study transmission in both pathways.

*Electrophysiological Procedures*

Procedures were the same for all rats in both Day 1 and Day 9 groups. Two rats, one control and one predator stressed were studied each day. Order of physiological testing was counterbalanced between controls and stressed rats over days. Rats were anaesthetized with urethane (1.5 g/kg) given in three divided doses separated by 10 minutes. Rats were placed in a stereotaxic instrument and injected under the scalp with 0.5 ml of marcaine (2% with epinephrine) for local anesthesia and to reduce bleeding. After scalp incision and retraction, holes were drilled in the

skull for electrode placement. In addition, skull screws were placed over the olfactory bulb to serve as a ground and references. Stimulating electrodes were Plastic One twisted bipolar stainless steel (0.125 mm in diameter, 20-30 k ohm impedance measured at 1000 Hz) aimed at VAB, BLA or ACe (Day 1 study only) in both hemispheres. The recording electrodes were Fredrick Haer stainless steel microelectrodes (1 um tip diameter, 0.5-1.5 ohm impedance measured at 1000 Hz) aimed at the BLA or PAG. The target brain area depended upon the study.

Rats were placed in a wooden box shielded with copper mesh. Temperature was maintained between 36-37°C by a rectal thermistor connected to a digital thermometer and feedback control to a DC heating pad (Frederick Haer) under the rat. Stimulation was biphasic with balanced constant current pulses (WP Model 601 photon coupled constant current stimulator) delivered at a rate of 0.5 Hz. At this rate, there was no decline in peak height of evoked potential over the 10 stimulations at a given intensity. Recording was differential between the microelectrode and the skull reference. Grass P15 capacitor coupled amplifiers (filters 10 Hz – 3 kHz) were used for preamplication. Amplification was increased via a Tectonics oscilloscope. The output from the oscilloscope then traveled to AD converters of an EMPAC 386 desktop computer. DataWave software controlled stimulus and sampling. Sampling of evoked potentials was at a rate of 10 kHz for 80 msec for each evoked potential.

Single pulses (0.5 Hz) were applied at varying pulse widths at 1.5 mA peak current to find a minimal pulse width which was sufficient to evoke a reliable potential. Vertical plane positions of stimulating and recording electrodes were adjusted to maximize the evoked potential. Once the desired positions of both the

electrodes and microelectrodes were determined, an equilibrium period of one half hour elapsed before stimulation began.

Both intensity series and double pulse series were applied to rats in both the Day 1 and Day 9 groups. Both the right and left hemispheres were tested and the order of left/right was counterbalanced over rats. Intensity series involved recording 10 evoked potentials at ten intensities (ranged from 250 mA to 2.5 mA in 250 μA steps). The potentials obtained at each intensity were averaged and analyzed. The double pulse series began ten minutes after the intensity series. The double pulse procedure was done in all groups except the Day 1 ACe-PAG. Intensity used was 1.25 mA with two interpulse intervals, 20 and 30 msec. This was done to estimate if group differences might be due to presynaptic mechanisms (Maren & Fanselow, 1995). Ten samples at each of the two interpulse intervals were taken, averaged and analyzed.

When the recording was finished, lesions were made with an anodal current passed between the tips of the stimulating electrodes and the microelectrodes to mark tip location (500 μA for 4 sec). Rats were given an overdose of sodium pentobarbital (Somnotol, 150 mg/kg) and perfused with cold phosphate buffered heparinized saline (PBS), followed by 4% paraformaldehyde in PBS and 0.5% potassium ferrocynanide and 0.5% potassium ferricynanide. This produced a blue dot at the tip of the electrodes. Brains were removed, sunk over night in 20 % sucrose, frozen in isopentane that was cooled by liquid nitrogen and kept at –70°C until sectioned.

*Histological Verification of Electrode Locations*

Frozen sections (40 μm) were taken through the electrode track and blue dot regions of the brain. The tissue was mounted on coated slides and stained with metachromatic cresyl violet. AP plane location was determined by counting sections from the decussation of the anterior commissure, AP –0.26 from bregma (Paxinos & Watson, 1982), to the electrode track. The number of sections was then multiplied by the section thickness which equaled the AP plane position to the nearest 40 μm.

An imager analyzer was used to determine the stereotaxic coordinates of the tip. The brain section being analyzed was normalized to the nearest corresponding rat atlas section. Normalizing factors were found by dividing the width of the rat section by the width of the same cross section through the atlas. The normalizing factor was then multiplied by the vertical and lateral position of the electrode tip. Two way ANOVAs for Day 1 and Day 9 groups were done separately examining Group (G) and Hemisphere (H) factors for AP.

*Electrophysiological analysis Methods*

The same analysis was completed on both Day 1 and Day 9 groups. In addition, only data from animals with electrodes in the appropriate locations were analyzed.

*Ratio Peak Height – Intensity Series*

The peak height of the evoked potential was calculated by computer from the averages at each intensity. Peak height (y) at all intensities was divided by threshold

peak height to yield ratio peak height. Raw peak height at each stimulus intensity was expressed as a ratio of peak height observed at threshold intensity in a given pathway for each rat. This controlled for differences in recording parameters. Threshold to evoke a potential in all pathways was calculated from the intensity series. This was done by fitting logistical dose response functions ($y=a+b/(1+(x/c)d$) to the plots of peak height versus intensity of stimulation with the Table Curve program (Jandel) for each rat, as has previously done in cats (Adamec, 1999). Y is peak height, x is intensity of stimulation, a, b, and c are constant parameters estimated in the fit. This function is derived from the rectangular hyperbolic function used to describe binding of a ligand to its receptor. The plots were smoothed (Jandel FFT smoothing algorithm) making sure that smoothing did not distort the trends visible in the raw data. All fits were good (all degrees of adjusted $r^2 > 0.90$). Threshold was calculated from the fitted function by determining the intensity which corresponded to either the function minimum or 18 μV, if the function minimum was less than 18 μV. Eighteen μV was chosen, as it was twice the noise value.

*Ratio Peak Height – Double Pulse Studies*

Peak height was taken by computer from averages at each interpulse interval. Ratio peak height was determined by raw peak height at pulse 2 divided by raw peak height at pulse 1 (P2/P1 ratios) because parameters of stimulation were constant over pulses.

*Bmax*

Bmax refers to the maximum number of receptors bound by a neurotransmitter. The logistical dose response curve may be used to estimate Bmax. Bmax = b, y = peak height and a and c are constants (equation above). Bmax was estimated from the fits of the logistical dose response curve to mean ratio peak height over intensity for control or predator stressed rats. Fitting was done with the Table Curve program (Jandel). Bmax scores were compared via t tests.

The degree to which Bmax estimates actual postsynaptic receptor binding depends on at least four assumptions. Firstly, peak height measurements estimate the physiological effects of neurotransmitter binding in a steady state condition and are monotonically related to those effects. Secondly, concentration of neurotransmitter is also monotonically related to intensity of stimulation. Thirdly, the maximal intensities used to stimulate those pathways achieve saturation of the transmission system being activated. Finally, the electrophysiological effects are a result of changes at the postsynaptic receptor.

*Behavioral Testing*

Anxiety like behavior was tested using the hole board, plus maze, light/dark box and acoustic startle. Blanchard, Blanchard, De Padua Carobrez, Veniegas, Rodgers & Shepherd, 1992 have also used the light/dark box as a measure of anxiety like behavior in rats. Testing took place between 8:45 AM and 10:15 AM. All tests were videotaped and measures were taken from the videotape as experimenters were hidden from view during testing.

*Hole Board*

The hole board test was used to measure activity and exploratory behavior. The hole board apparatus was a square wooden box measuring 60 cm on a side with walls that were 35 cm above the floor of apparatus. The floor of the apparatus was elevated 12 cm above ground and had four evenly spaced holes with each hole large enough for the rat's head. The holes formed a square that was 14 cm from the walls of the box. The hole board was painted flat gray.

*Hole Board Behavioral Measures*

Six measures were obtained from this apparatus; frequency of rearing, time spent moving, number of head dips, time spent near wall, time spent in center and number of fecal boli. Both frequency of rearing and time spent in motion (time active) were a measure of activity. Exploratory behavior was measured with head dips (placing the snout or head into a hole). Time spent near the wall was a measure of thigmotaxis. A rat was considered near a wall when its four feet were in the space between the holes in the floor and the wall.

*Elevated Plus Maze*

The elevated plus maze was used in conjunction with the hole board as an independent measure of activity and exploratory behavior. The maze consisted of four arms arranged in the shape of a plus sign. Each arm was 10 cm wide and 50 cm long and joined in the center of the maze by a 10 cm square platform. Two of the

four arms had walls that rose 40 cm above the floor of the apparatus (closed arms). The remaining two opposing arms had a 3 cm high edge running around the periphery to prevent the rats from falling off (open arms). The maze was elevated 50 cm off the ground and painted flat gray.

Rats were first placed in the hole board for five minutes. The rats were then removed and transferred by gloved hand to the elevated plus maze for a further five minutes of testing. Rats were then returned to their home cages.

*Elevated Plus Maze Behavioral Measures*

Many behavioral measures were examined 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.

Other measures of exploration included head dips (placing the snout or head over the side of the open arm) and rearing. These behaviors were divided into three types; protected (rat had all its 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.

Cautious Exploration was scored as stretch attends and flat back approaches. A stretch attend occurred when a rat stretched its body forward and either sniffed or

visually scanned. Flat back approach emerged from a stretch attend posture with forward locomotion with the back concave and the stomach near the floor of the maze. Again, stretch attends and flat back approaches were scored as protected, unprotected or center.

Measures of anxiety like behavior 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.

Measures of risk assessment were also taken to assess anxiety like behavior. Blanchard & Blanchard (1989) scored risk assessment when a rat poked its head and forepaws into an open arm of the maze. The rat's hindquarters must be in a closed arm of the maze. Frequency of risk assessment was also measured. Finally, a relative risk assessment was calculated by dividing the frequencies by the time spent in the closed arms. Fecal boli deposited in the maze were also counted.

*Light/Dark Box*

This apparatus was used to assess anxiety like behavior in rats. The light/dark box was a single alley made with 0.5 inch plywood divided into two separate chambers of equal size. Each chamber was 31.75 cm long, 10.48 cm wide and 14.06 cm high. Both chambers were covered with a transparent Plexiglas top hinged so it

could be opened. Ventilation was provided by cuts made in the center pieces. One of the chambers was painted white with a wooden floor while the other chamber had a metal mesh floor and was painted black with a black plastic covering on the top. In addition, a 100-watt lamp was positioned 660 cm above the white chamber which produced a light intensity in the center of the floor of 55 foot candles. The dark chamber, however, had an intensity of only 2 foot candles at the center of the floor. A video camera was mounted directly above the apparatus for later analysis.

Testing began when the rat was placed in the light/dark box in the white chamber facing away from the dark chamber. The rat was allowed to explore both chambers freely for five minutes. There were two boxes used to allow the testing of one control and one stressed rat at the same time. Boxes used for testing were counterbalanced across groups.

*Light/Dark Box Behavioral Measures*

Time spent in the light and dark chambers and number of entries into either chamber was measured. A rat was considered to be in a chamber when all four feet were within its boundaries. Time spent between the two chambers was calculated as 300 sec (5 minutes) minus time in both chambers. Latency to escape from the lighted chamber after initially being placed in it was also measured. Finally, number of fecal boli deposited in either chamber was also counted.

*Acoustic Startle Chamber*

Unconditioned startle response to an acoustic stimulus was examined using a standard startle chamber (San Diego Instruments). A 20.32 cm Plexiglas cylinder was used to hold the animal inside the apparatus. In addition, the chamber was outfitted with a speaker for producing 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.

*Acoustic Startle Measures*

Animals were acclimated to the apparatus for ten minutes prior to testing. Upon testing, rats were given 20 trials (1 per minute) of 20 msec bursts of 120 db of white noise rising out of a background of 60 db. A computer recorded 20 samples of transducer output. Samples included a 20 msec baseline and 250 msec sample after onset of the noise burst. Baseline (V Start) was considered to be the average transducer output prior to the noise burst. Peak startle amplitude within each sample (V max) was found and expressed as Vmax – Vstart for analysis. At the end of this test, the rat was returned to its home cage.

*Statistical Analysis*

All data were analyzed using an ANOVA design. Planned comparisons were done using t tests and other multiple comparisons were done using Bonferroni protected t tests.

# Results

## PCREB Study

### *PAG*

Raw pCREB densitometry data of each column in each hemisphere were converted to optical density (OD) units relative to the whole section. This was done by converting the raw PAG and raw whole section 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. Analysis was performed on the average ratio of OD values in particular PAG areas to average OD values for the entire section. Data were analyzed with a three way mixed ANOVA assessing Group, Hemisphere, and Column with repeated measures on Hemisphere and Column. Significant main effects were found for Side ($F\{1,14\}=11.04$, $p < .001$) and Column ($F\{2, 28\}=36.28$, $p<.000001$). There was no main effect of Group ($F\{2,14\}=0.27$, $p>.05$). There were two significant two-way interactions: Group by Hemisphere ($F\{2,14\}=4.9$, $p<.05$) and Hemisphere by Column ($F\{2,28\}=6.79$, $p<.005$). The Group by Column interaction was not significant ($F\{4,28\}=1.11$, $p>.05$).

More importantly, there was a three-way interaction with Group x Hemisphere x Column ($F\{4,28\}=5.93$, $p<.005$). Mean contrasts were done to

determine the nature of the interaction using Bonferroni protected t tests. Planned comparisons were made between the three groups (control, restrained and predator stressed) for each column and in each hemisphere. **Figure 2** displays the results of these comparisons. The handled control and restraint groups show equal and lower intensity of pCREB staining than the predator stressed group in the right lateral column. There were no differences between groups in the right hemisphere in the dorsal or ventral columns. In the left hemisphere, however, there were differences in all three columns. The predator stressed and handled control had equal and higher intensity of pCREB staining than the restrained controls in both the ventral and lateral columns. In addition, the handled control showed more staining than both the restraint and predator stressed rats in the left dorsal column.

*Amygdala*

For the amygdala, relative OD in each hemisphere for each nucleus was calculated as a ratio of a rectangular portion of the internal capsule on the same side. The internal capsule was used as the divisor for each nucleus. The internal capsule was used because it provided an estimate of background staining. A three way ANOVA was performed assessing Group, Hemisphere, and Nucleus with repeated measures on Hemisphere and Nucleus. Only the Nucleus main effect was significant ($F\{5,45\}= 29.44$, $p<.000001$). Neither Group ($F\{2,9\}=1.85$, $p>.05$) nor Hemisphere ($F\{1, 8\}=4.48$, $p>.05$) main effects were significant. The only significant two-way interaction was Group by Nucleus ($F\{10,45\}=2.63$, $p<.05$). Neither the Group x Hemisphere ($F\{2,9\}=.27$, $p>.05$) nor Hemisphere x Nucleus ($F\{5, 40\}=1.14$, $p>.05$)

interactions were significant. In addition, the three-way interaction of Group x Hemisphere x Nucleus was not significant ($F\{10,40\}=1.84$, $p>.05$).

Given the Group x Nucleus significant interaction, mean contrasts were done between groups across nuclei, collapsed over hemisphere, for each amygdala nucleus (**Figure 3**). Both the MeAV and ACo had similar staining patterns of handled controls with the least, then restrained controls then predator stressed with the most intense pCREB staining. The handled control and restraint groups had equal and lower levels of pCREB staining than the predator stressed group for all other nuclei (ACe, BMA, BLA, and LA).

### *VMH*

The VMH was analyzed relative to the same internal capsule area as was the amygdala. A two way ANOVA assessed Group and Hemisphere with Hemisphere as the repeated measure. There were no significant main effects or interactions.

## Electrophysiological Studies

### *Histological Results*

In the Day 1 study, 12 control and 12 predator stressed were on target bilaterally for VAB-BLA and for ACe-PAG pathways. The bilateral on target placements for the Day 9 study were 4/10 controls and 6/10 predator stressed for the VAB-BLA pathway and 5/10 controls and 7/10 predator stressed in the BLA-ACe pathway. A two way ANOVA was done on AP, lateral and vertical plane values for

on target rats assessing Group and Side differences with repeated measures on Side. The analysis was done separately on stimulating and recording electrode placements for Day 1 and Day 9 rats. There were no main effects or interactions.

Analysis was done to determine whether placement of stimulating or recording electrodes differed between Day 1 and Day 9 rats. A two way ANOVA was done assessing Day 1 and Day 9 rats as separate groups (collapsed across stressed and control groups), and Side, with repeated measures on Side. The PAG placements showed no differences in AP and lateral planes. However, there was a significant difference in vertical plane ($F\{1,20\}=6.05$, $p<.024$). Tips of the PAG electrodes of Day 1 rats fell at 5.36 +/- 0.10 mm below bregma, while Day 9 rat electrodes were deeper at 5.66 +/- 0.06 mm below bregma. Placements of the electrodes of both the Day 1 and Day 9 can be seen in **Figure 4.** The VAB placements did not differ in the lateral or vertical planes. However there was a significant difference between the Day 1 and Day 9 rats in AP plane ($F\{1,20\}=5.29$, $p<.03$). Day 1 rats were more anterior than Day 9 rats (-6.14+/-.15 vs. -6.66+/-.16, mean +/- SEM mm posterior to bregma, Day 1 vs. Day 9). BLA placements differed in two planes between the Day 1 and Day 9 rats (all $F\{1,20\}>12.55$, $p<.01$). The Day 1 electrodes were more anterior (-3.14+/-.09 vs -3.96 +/-.10, mean +/- SEM mm posterior to bregma) and less deep (8.49 +/-.08 vs. 9.12 +/-.08, mean +/- SEM mm below bregma, Day 1 vs. Day 9). In both groups, however, recording electrodes were in the posterior basolateral amygdala.

## Electrophysiology Results

*Threshold, Peak Height and Stimulus Intensity*

Thresholds were converted to intensity in μC (current x width). Two way ANOVAs, assessing Group and Hemisphere effects with repeated measures on Hemisphere were done on both threshold intensity and threshold peak height. This was done in the control and predator stressed rats for both the Day 1 and Day 9 studies. There were no effects; thus one can conclude that differences in ratio peak height cannot be attributed to size of the divisors. In addition, differences between groups were not accompanied by differences in the threshold to evoke potentials. Finally, intensity of stimulation for the intensity series used on each rat was converted to μC. A three way ANOVA was performed assessing Group, Hemisphere, and Intensity with repeated measures on Hemisphere and Intensity. Again, there were no differences, which implies that differences in groups cannot be ascribed to intensity of stimulation differences because of differences in pulse width.

The VAB-BLA pathway was examined in both the Day 1 and Day 9 studies. Comparisons of intensity of stimulation and peak height at threshold were made across studies. There were no differences between Day 1 and Day 9 on intensity of stimulation. However threshold peak height differed ($F\{1,66\}=10.16$, $p<0.002$) with threshold peak height larger in the Day 1 than in Day 9 rats (176.8 +/-25.6μV vs. 42.3 +/-12.8 μV respectively). The difference in peak height may be due to the small variations in location of the VAB and BLA electrodes between Day 1 and Day 9.

## Intensity Series

A three way ANOVA was performed on ratio peak height data separately for each study assessing Group, Hemisphere and Intensity with repeated measures on Hemisphere and Intensity.

*VAB-BLA Pathway*

There were Group x Hemisphere x Intensity interactions for both Day 1 ($F\{9,198\}=12.16$, $p<.0001$) and Day 9 ($F\{9,72\}=3.3$, $p<.002$) analyses. Interactions appear in **Figure 5**. A similar pattern over intensity was seen in both the Day 1 and Day 9 studies. In the left hemisphere, relative to control, there was a suppression of ratio peak height in predator stressed rats, whereas the opposite was seen in the right hemisphere. Left hemisphere suppression is seen over intensities 2-10 in the Day 1 study but covers a narrower range in the Day 9 study (intensities 9 and 10). In contrast, predator stressed rats show elevations of response in the right hemisphere as compared to control in both Day 1 and Day 9 studies over intensities 3-10.

*ACe-PAG Pathway (Day 1 study only)*

There was a Group x Hemisphere x Intensity interaction ($F\{9,198\}=4.86$, $p<.0001$), see **Figure 6**. Predator stressed rats showed stronger responses than control in the PAG to ACe stimulation over intensity in both hemispheres. In the left hemisphere groups differed from intensities 6-10, whereas in the right hemisphere, groups differed over intensities 3-10.

*BLA-PAG Pathway (Day 9 study only)*

There were Group x Hemisphere x Intensity interactions for BLA-PAG ($F\{9,54\}=2.91$, $p<.005$), see **Figure 7**. For predator stressed rats, the BLA-PAG response was suppressed relative to controls over intensities 2-10 in the left hemisphere. In contrast, groups did not differ in right hemisphere response.

## Double Pulse Series Results

A three way ANOVA was conducted on P2/P1 data for each study separately. Analysis of variance assessed Group, Hemisphere and Interpulse Interval effects with repeated measures in Hemisphere and Interpulse Interval.

*VAB-BLA Pathway.*

For Day 1 data, there was a Group x Hemisphere interaction ($F\{1,22\}=9.12$, $p<.007$) and an Interpulse Interval effect ($F\{1,22=26.23$, $p<.001$). There were no significant effects in the analysis of Day 9 data. Data for Day 1 and Day 9 appear in **Figure 8**. Both controls and stressed rats showed equal P2/P1 ratios in the right hemisphere for both Day 1 and Day 9 studies. In the left hemisphere (Day 1 study), however, control rats showed elevated P2/P1 ratios relative to stressed rats. Day 9 data did not show this difference which may be related to factors responsible for the lessening response of this pathway over intensity on Day 9.

*BLA – PAG Pathway (Day 9 only).*

A double pulse analysis was performed on the left hemisphere data only because there were no differences between groups in the right hemisphere over intensity. There was a Group x Interpulse Interval interaction ($F\{1,11\}=4.86$, $p<.05$), which appears in **Figure 9**. Control rats displayed suppressed P2/P1 ratios relative to stressed rats but this difference only appeared in the 20 msec interpulse interval.

## *Bmax Analysis*

For pathways showing group differences, Bmax was estimated from the intensity series data as described in the Methods. The size of Bmax was compared between groups using t-tests.

*VAB-BLA Pathway.*

Results were similar for both Day 1 and Day 9 studies and can be seen in **Figure 10** (all $t\{12\}>8.75$, $p<.001$). In the right hemisphere, predator stressed rats showed greater Bmax while in the left hemisphere; control rats showed greater Bmax.

*ACe-PAG Pathway (Day 1 study only).*

There were differences in Bmax between control and predator stressed rats in both hemispheres (all $t\{12\}>3.75$, $p<.003$; **Figure 11**). Specifically, stressed rats showed larger Bmax than controls.

*BLA-PAG Pathway (Day 1 study only).*

There were no differences between controls and stressed rats in estimated Bmax.

## Behavioral Results for Day 9 Study Groups

One way ANOVAs contrasting handled control and predator stressed groups on all behavioral measures were done. This analysis was done for rats with VAB-BLA and BLA-PAG on target bilateral electrodes. Only results that were significant will be reported.

*Hole Board Test.*

There were no group differences on any measure in this apparatus. This is consistent with past experiments (Adamec, 1997) and suggests that changes in plus maze exploration are not due to changes in activity or exploratory tendency per se.

*Elevated Plus Maze*

There were group differences for seven of the measures (all $F\{1,8\}>5.73$, all $p<.044$ for VAB-BLA and all $F\{1,10\}>6.80$, all $p<.03$ for BLA-PAG groupings). Predator stressed rats were more "anxious" which was evident by the typical measures of anxiety-like behavior; ratio-time and entry, risk assessment, open arm

avoidance (lower ratio time and entry reduced relative time risk assessment. Adamec (1997) has reported similar results.

Measures of exploration also changed. Stressed rats entered the closed arms less frequently and showed fewer total arm entries than controls (**Figure 12**). This suppression may be due to increased anxiety-like behavior. If one removes the influence of ratio time and ratio entry from arm entry measures with analysis of covariance, the group differences disappear (*e.g.* $F\{1,6\}<1.36$, $p<.28$ for VAB-BLA). Predator stressed rats also show decreases in both unprotected head dips and unprotected stretch attends as compared to controls. As with arm entries, covarying effects of ratio time and entry from head dips and stretch attends removed the group effects ($F\{1,6\}<1.65$, $p<.24$).

*Light/Dark Box*

Latency to escape from the lighted chamber differed in control and predator stressed rats ($F\{1,8\}=6.24$, $p<.04$; $F\{1,10\}=6.26$, $p<.032$ for VAB-BLA and BLA-PAG, respectively. Predator stressed rats escaped more quickly than controls (**Figure 13**).

*Acoustic Startle*

Data were not normally distributed; (Omnibus normality tests , all $t >26.06$, $p<.001$) therefore group differences were assessed with non-parametric tests (Mann Whitney U tests). Predator stressed rats (VAB-BLA and BLA-PAG) showed larger startle amplitudes than control rats over all startle trials (all $p<.01$, **Figure 14**).

# Discussion

The goal of this study was to examine the neural basis of lasting changes in affect produced by a severely stressful event. This is a relevant topic as approximately 15 % of people who experience a stressful event develop Post Traumatic Stress disorder (Kessler et al, 1995). Research in humans has implicated the limbic system in PTSD. Specifically, Vietnam veterans suffering from PTSD show an increase in PET activation of the right amygdala in response to stimuli that remind them of their combat trauma (Shin et al., 1997).

The importance of the right hemisphere for long-lasting responses to stress has also been reported in cats, and now in rodents. This project investigated the neural transmission between areas involved in stress and anxiety and pCREB expression in those brain areas.

## Behavioral Response to Predator Stress

Behavioral changes reported in the Day 9 electrophysiological study were consistent with previous work. Specifically, increased open arm avoidance, decreased risk assessment, increased lighted chamber avoidance and increased acoustic startle response have all been reported following predator stress (Adamec et al., 1998; Adamec et al., 1999; & Adamec et al., 1999). The startle results are particularly important as patients with PTSD have demonstrated an exaggerated

response to startle stimuli (Kolb, 1987; Butler et al., 1990). There were no differences in behavior between control and exposed rats in the hole board which suggests that changes in plus maze exploration were not due to alterations in activity or exploratory behavior, again replicating previous work (Adamec, 1997, Adamec et al., 1999).

## Potentiation of Transmission and Predator Stress

There are two reasons why the electrophysiological differences observed in the present study are likely to reflect differences in potentiation of transmission over the pathways investigated. First, studies of electrically induced LTP in cortex show potentiation of response over a fixed intensity series of stimulation (Heginbotham & Dunwiddie, 1991). Second, studies of stress induced LTP of amygdala efferent transmission also find potentiation of intensity series response (Adamec, 1998). Moreover, potentiation in amygdala efferent transmission occurs without evidence of change in threshold to evoke field potentials in efferent targets such as the PAG (Adamec, 1998). In the present study, there were no differences between controls or predator stressed groups in either threshold to evoke field potentials, amplitudes at threshold, or intensity of stimulation used in the intensity series. The differences between controls and predator stressed rats can therefore be interpreted as a form of potentiation of transmission over the pathways investigated. Nor can the differences observed in response between groups and hemisphere be due to differences in location of stimulating and recording electrodes.

## Hemispheric Biases in Transmission

The goal of this study was to gain a better insight into the neural substrates of lasting change in affect produced by a highly stressful event (predator stress). The electrophysiological studies performed were inspired by work on felines. Overall, results from this study are consistent with those in cats. Specifically, changes in neural transmission in amygdala efferent pathways in both hemispheres are important in stress induced lasting change in defensiveness (Adamec, 1997). The results of this study show the importance of potentiation of transmission in the right hemisphere for stress induced behavioral change. The significance of the right hemisphere is also seen in the cat, as LTP like changes in the right amygdalo-PAG pathway are involved in maintenance of lasting changes in affect (Adamec, 1997).

Changes in transmission in the left hemisphere are also seen in both felines and rodents. In the cat left hemisphere, LTP of efferent transmission from the amygdala to medial hypothalamus and amygdala to PAG covary with defensiveness increased by either partial kindling or FG-7142 (Adamec, 1997). LTP in these pathways is transient, however, diminishing to baseline, though increased defensiveness persists (Adamec, 1997). In addition, LTP in amygdala efferents in both hemispheres contributes to initial increases in defensiveness, but lasting changes in behavior depend on LTP in the right hemisphere (Adamec, 1997; Adamec, 2000). In this study, both right and left hemispheres effect anxiety like behaviors in rats. The results, however, vary slightly from cat data, as both potentiation and depression

are seen in the left hemisphere, depending on the pathway. The specific pathways will be discussed in detail in the following sections.

*ACe-PAG Pathway*

Transmission in amygdala efferents in both rodents and felines is quite similar. Predator stress potentiates transmission in the ACe-PAG pathway in both the left and right hemispheres for at least one day post predator stress. Similarly, bilateral potentiation of amygdalo-PAG transmission associated with increased defensiveness has been seen in the cat following kindling and administration if FG-7142 (Adamec, 1997; Adamec, 2000; Adamec, 1998). Another similarity is the strength of potentiation in the right hemisphere of both rodents and felines. In this study, potentiation of transmission appears stronger in the right hemisphere, in that it is apparent at a lower range of intensities (Figure 6). Likewise, in the cat, LTP of left amygdalo-PAG transmission is not as strong in that it is shorter lived than in the right hemisphere (Greisen, Sheikh, Bolwig & Mikkelsen, 1997; Adamec, 1998).

*BLA-PAG Pathway*

Unlike the ACe-PAG pathway, a depotentiation of transmission in the left hemisphere is seen in the BLA-PAG pathway (Figure 7). As noted above, there is a potentiation in the left hemisphere in the ACe-PAG pathway at one day post predator stress. Unfortunately, there are no data for the behavior of this transmission at Day 9, although it may persist until day nine. It is difficult to understand the difference in left hemisphere transmission between the efferent pathways to the PAG (Ace-PAG,

potentiation; BLA-PAG, depotentiation). There is an efferent pathway from the BLA through the ACe to the PAG (Gomez, Chandler & Behbehani, 1996). If this were the route used in the BLA-PAG transmission in this study, one would expect to see longer latency to onset in the BLA-PAG as compared to the ACe-PAG. However, this was not the case. There was a slight difference, but not significant, which may be due to the greater anatomical distance. In addition, there was a significant difference in location of PAG recording electrodes in the two pathways. Because of both the difference in location of recording electrodes and no difference in latency of onset between the two pathways, the data suggest that BLA-PAG may be measuring an afferent to the PAG independent of the ACe-PAG.

*VAB-BLA Pathway*

There were data available for both the Day 1 and Day 9 studies in this pathway. Both the Day 1 and Day 9 predator stressed rats showed potentiation in the right VAB-BLA and depotentiation in the left VAB-BLA. In the left hemisphere, however, the differences between groups were only apparent at the higher intensities in the Day 9 study as compared to the Day 1 study. This may reflect a change in stressed rats over nine days or it may simply be the slight differences in location of stimulating and recording electrodes in Day 1 and Day 9 rats.

Overall, it is apparent that there is a right hemisphere bias in potentiation of amygdala afferent pathways following predator stress. In addition, there appears to be a decrease in transmission in the left hemisphere of predator stressed rats as compared to control. As is the case for all pathways, longitudinal studies in awake

and freely moving rats need to be done in order to determine the effects of stress over time.

The results in this pathway are interesting in view of data implicating NMDA receptors in predator stress induced behavioral change. As discussed above, systemic and amygdala NMDA receptor block prevents predator stress effects on rodent affect (Adamec et al., 1999; Adamec et al., 1999). Moreover, the VAB-BLA pathway supports NMDA dependent LTP associated with contextual fear learning (Maren, De Oca, & Fanselow, 1994; Maren & Fanselow, 1995). These results suggest that the VAB-BLA pathway may employ NMDA dependent neural changes which mediate aspects of increased affect following predator stress.

## Mechanisms of Potentiation

*Pre-synaptic Mechanisms*

Paired-pulse methods were employed to investigate whether changes in VAB-BLA and BLA-PAG were a result of a pre-synaptic change (Maren & Fanselow, 1995). Paired-pulse data are not available for the ACe-PAG pathway. Electrically induced LTP in the VAB-BLA pathway reduces paired-pulse facilitation (Maren & Fanselow, 1995) yet there was no evidence of such a reduction at day 9 in the present study. In the VAB-BLA pathway in the Day 9 study (Figure 8), potentiation in the right hemisphere and depotentiation in the left hemisphere of stressed rats was not accompanied by changes in paired-pulse facilitation relative to controls. Differences in paired-pulse response were seen only at one day after predator stress, and only in

the left hemisphere. Therefore, changes in right hemisphere VAB-BLA transmission may not involve presynaptic mechanisms. Depotentiation in the left hemisphere in stressed rats at Day 1 was accompanied by a paired pulse response of 1.0, which was less than an apparent paired pulse facilitation in controls. Since paired pulse facilitation in other systems involves accumulation of calcium in the presynaptic terminal (Dittman & Regehr, 1997), it is possible that stress may be reducing this accumulation in the left hemisphere, but only at Day 1. This mechanism cannot account for the lasting differences in the left hemisphere between control and stressed rats at Day 9. Therefore, a presynaptic mechanism cannot account for the lasting differences between stressed and control rats in the left VAB-BLA either.

In Day 9 rats, there was no evidence of paired-pulse facilitation or depression in the BLA-PAG pathway in predator stressed rats relative to control at the 30 msec interpulse interval. There was, however, paired-pulse depression in controls relative to predator stressed rats at the 20 msec interpulse interval (**Figure 9**). This suggests a presynaptic LTP-like potentiation in controls in the left hemisphere. These differences, however, are only evident at the 20 msec interpulse interval. Predator stress may therefore be presynaptically depotentiating this naturally potentiated state in the controls. Data described in the following section suggest a post synaptic process in all but the BLA-PAG pathway.

*Post-synaptic Mechanisms*

Potentiation by predator stress in both the VAB-BLA and ACe-PAG pathways in the right hemisphere was associated with increases in Bmax. The right VAB-BLA

pathway demonstrated increases in Bmax at both Day 1 and Day 9 post predator stress. Adamec & Young (2000) have also shown long lasting increases in Bmax associated with potentiation in amygdala efferent pathways in the cat. As described above, Bmax refers to the maximum number of receptors bound by neurotransmitters. Increases in Bmax suggest that a change in post synaptic receptor number may underlie the potentiation. These changes in receptor number may reflect: increased numbers of post synaptic receptors, changes in synaptic morphology to increase post synaptic receptor sites, as in perforated synapses in LTP (Geinisman, de Toledo, Morrell, Heller, Rossi & Parshall, 1993) or synaptogenesis.

The left hemisphere in the VAB-BLA pathway showed a reduction in Bmax in predator stressed rats (Figure 10). This suggests a reduction in receptor number which mediates the reduced transmission in this pathway following predator stress.

## PCREB and Predator Stress

As discussed above, cannulation of MK-801 into the amygdala 30 min prior to cat exposure blocks behavioral change, but which behaviors are blocked depends on the hemisphere in which NMDA receptors are blocked (Adamec et al., 1999). Pathways were investigated which appear to support glutamate based LTP (*e.g.* VAB-BLA) (Adamec, 1993). For these reasons, pCREB induction following predator stress in rats was examined. CREB and pCREB are linked to gene transcription changes involved in maintenance of LTP (Dash & Moore, 1996) and phosphorylation of CREB is regulated by NMDA receptors (Das, Grunert, Williams & Vincent

(1997). Therefore visualization of pCREB may be helpful in identifying putative neural circuitry mediating behavioral change in which NMDA dependent LTP is induced.

PCREB expression was analyzed in the PAG, amygdala and the VMH. As stated above, the analysis examined hemispheric differences in all three brain areas for predator stressed, handled control and restraint groups. In addition, the PAG was divided into lateral, dorsal, and ventral regions and the amygdala was sub-divided into its nuclei for analysis.

*PAG, pCREB & Predator Stress*

The PAG results confirm the right hemisphere's involvement in predator stress. The right lateral column of the PAG showed elevated pCREB LIR in predator stressed rats as compared to the handled control and restraint rats (Figure 2). To confirm the importance of the right lateral column, studies need to be done which block pCREB expression in the right lateral column. After which, behavioral tests need to be completed which compare pCREB blocked rats and controls.

As stated above, there is potentiation in the right ACe-PAG pathway. The results of the pCREB study (increased pCREB LIR in the right lateral column of PAG), in combination with the electrophysiological study, suggest the importance of LTP in the PAG of the right hemisphere in predator stress.

These findings are consistent with previous data in the cat and rat. Adamec (1997) has shown that, in the cat, LTP in the right amygdala-PAG pathway produced by kindling or pharmacological stress is more persistent than LTP in the left

hemisphere. In addition, behavior changes as a result of kindling or pharmacological stress do not disappear with spontaneous decline of the potentiation in the left amygdala-PAG pathway. Moreover, low frequency stimulation (LFS) of the amygdala depotentiated LTP in the right, but not left, amygdala-PAG pathway and reversed the behavioral effects of ventral hippocampal kindling (Adamec, 1999). Finally, LFS in the rat PAG has been shown to reverse the behavioral effects of predator stress (Adamec, 2001). These data support the view that an LTP-like mechanism in the PAG may be involved in behavioral changes in rats following predator stress.

As was discussed previously, pCREB is involved in the maintenance of LTP (Silva *et al.*, 1998). The increase in intensity of pCREB staining in the right lateral column of the PAG might suggest that there would be a longer lasting potentiation of transmission in the right PAG following predator stress paralleling findings in the cat. Further work is needed to test this hypothesis.

*Amygdala, pCREB LIR and Predator Stress*

The amygdala was divided into six separate nuclei for analysis: ACe, BM, LA, BLA, MeAV, and ACo. The handled and restraint groups both showed pCREB staining in the ACe, BM, LA and BLA that was equal and lower to that of the predator stressed group (**Figure 3**). The MeAV and ACo nuclei, however, did not follow this pattern. The handled group displayed the lowest pCREB staining, the restraint group displayed a medium amount of staining and the predator stressed group had the highest intensity of pCREB staining (**Figure 3**). A possible

explanation for these results may be that certain nuclei (ACe, BM, LA, and BLA) may be specifically involved with predator stress. Both the control and the restraint groups have equal staining and differ from the predator stressed group. However, other nuclei (MeAV & ACo) may not be specifically associated with predator stress because there is increased staining in the restraint group as well as the predator stressed group. The predator stressed group does, however, have elevated pCREB LIR so more studies are required to confirm this hypothesis. For example, pCREB block of certain nuclei followed by behavioral testing on an apparatus already associated with predator stress may tease out the nuclei specific to predator stress.

Predator related stress has been shown to affect expression of other gene regulating proteins, including cFos. Increases in cFos expression are considered to be at least markers of neural activation. It is an empirical question whether cells expressing cFos in response to stress would be the same as those expressing pCREB. There are few data to date which directly address this question. Canteras and Goto (1999) report that cat exposure increased cFos-like immunoreactivity in the rostral two-thirds of the rat PAG, overlapping areas examined in this study. Expression of cFos was mostly seen in the dorsomedial and dorsolateral regions. At the caudal levels, however there was greater cFos expression in the lateral and ventrolateral area of the PAG. In addition, Dielenberg, Hunt and McGregor (2001) found that cat odor-exposed rats showed greater cFos expression than controls in the medial amygdala, medial hypothalamus and the PAG with no difference in the VMH. Again these findings are somewhat consistent with the data in this study, except that pCREB expression increased in other nuclei of the amygdala. The lack of correspondence

between Dielenberg et al. and the present data may reflect the nature of the stressor (odor as opposed to unprotected exposure to a cat) or the fact that cFos and pCREB expression do not necessarily overlap. In support of the latter possibility are the data of Stanciu, Radulovic and Spiess (2001), who studied patterns of cFos and pCREB expression in the brains of mice exposed to contextual fear conditioning. They found that pCREB and cFos expression followed different regional and stimulus-dependent patterns in conditioned mice. These data indicate that neuroplastic change associated with increased fear in association with a context produce very different patterns of expression of cFos and pCREB. The data also support the view that environmental events which induce pCREB and cFos expression in cells do not necessarily do so in the same cells.

## Conclusions

Results from the pCREB study show differences in pCREB expression depending on the group, hemisphere, column (PAG) and nuclei (amygdala). The PAG results indicate that the right lateral column is specifically involved in predator stress. More studies need to be undertaken which block pCREB expression and measure change in behavior to confirm the conclusions of this study.

The electrophysiological study provides evidence for hemispheric differences in amygdala afferent and efferent neural transmission following predator stress in rats. The stressor (cat), depending on the pathway and hemisphere produces both potentiation and depotentiation. There also appears to be a postsynaptic mechanism

mediating stressor-induced changes (in all pathways except BLA-PAG). These conclusions are tentative until replicated in chronic longitudinal studies taking pre and post stress measures in the same rat.

In conclusion, both the pCREB and electrophysiological results combined suggest a long-term neuroplastic change in both the amygdala and the PAG following predator stress. Not only is CREB a marker for neural activity, but CREB transcription appears to mediate synaptic restructuring (Silva *et al.*, 1998). Bmax, as described above, refers to the maximum number of receptors bound by a neurotransmitter. Both the ACe-PAG and VAB-BLA pathways show either an increase or decrease, depending on the hemisphere, in Bmax in predator stressed rats compared to controls (Figure 10 and Figure 11). Enhanced pCREB expression, as seen in the predator stressed rats, may therefore mediate remodeling of the synapse which is reflected in the changes in Bmax.

We have shown increased pCREB expression in the right hemisphere of the PAG and potentiation of transmission in the right amygdala to PAG pathway. Both the pCREB and electrophysiological experiments confirm the role of the amygdala and PAG in predator stress. As seen in cat and human data, these experiments support the importance of the right hemisphere in stress-induced changes in affective behavior.

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# Figure Captions

Figure 1. Photomicrographs of two sections from the PAG (AP plane positions 6.40 mm posterior to Bregma), one from the Predator Stressed group and the other from the Handled Control group. Sections were stained for pCREB-like immunoreactivity. There is some ventricular swelling in the Handled control section due to the freezing process.

Figure 2. Mean + SEM relative optical density units (PAG optical density units divided by total section optical density units) in all three columns in both hemispheres for each group (Handled control, Restrained control and Predator stressed) 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 (all $t(28)>3.45$, $p<.05$, Bonferroni protected 2 tailed t tests).

Figure 3. Mean + SEM relative optical density units (Amygdala optical density units divided by internal capsule optical density units) in all six nuclei (Handled control, Restrained control and Predator Stressed), collapsed across hemisphere, are graphed. Comparisons were made within a nucleus between groups. Means marked with the same letter do not differ but differ from those with different letters (all $t(28)>3.23$, $p<.05$, Bonferroni protected 2 tailed t tests). MeAV – medial nucleus; ACe – central nucleus; BMA – basomedial nucleus; BLA – basolateral nucleus; LA – lateral amygdala; ACo – cortical nucleus.

Figure 4. Mean +/- SEM in lateral and vertical planes of electrode tip locations of all on-target rats are projected onto sections from Paxinos and Watson (1982). AP plane by each section is in mm posterior to bregma. Abbreviations: Aq – aqueduct of Sylvius; AhiAL – anterolateral amygdalo-hippocampal area; Astr – amygdalo-striatal transition area; BLA – basolateral amygdala; BLV – ventral basolateral amygdala; BMA – anterior basomedial amygdala; BMP – posterior basomedial amygdala; BSTIA – intra amygdaloid division of the bed nucleus of the stria terminalis; CeC – capsular central amygdala; CeL – lateral central amygdala; CeM – medial central amygdala; csc – commissure of the superior colliculus; CA1,2,3 – fields CA1,2,3 hippocampus; DEn, VEn – dorsal, ventral endopiriform nuclei; DG – dentate gyrus; dlf – dorsal longitudinal fasciculus; DLPAG -, DMPAG, LPAG – are dorsolateral, dorsomedial and lateral periaqueductal gray; DMD, DMV – dorsal, ventral dorsomedial hypothalamus; EW – Edinger-Westphal nucleus; I – intercalated amygdala nucleus; LaDL – dorsolateral lateral amygdala; LAVM –

ventromedial lateral amygdala; LaVL – ventrolateral lateral amygdala; LH – lateral hypothalamus; IPR – rostral interpeduncular nucleus; MeAD, MePD, MePV – anterodorsal, posterodorsal, posteroventral medial amygdala; PH – posterior hypothalamus; Pir – piriform cortex; PLCo, PMCo – posterolateral, posteromedial cortical amygdala; RPF – retrorubal field; S – subiculum; SC – superior colliculus; SNR – substantia nigra reticulata; St – stria terminalis; Su3 – supraoculomotor cap; VMHC, VMHDM, VMHVL – central, dorsomedial, ventrolateral ventromedial hypothalamus; VTA – ventral tegmental area; 3 – oculomotor nucleus.

Figure 5. Means + SEM (or Means – SEM) of ratio peak height (PH) versus intensity of stimulation for potentials evoked in BLA by VAB stimulation. Separate plots are shown for predator stressed (exposed) and control rats for Day 1 and Day 9 studies both in right and left hemispheres. An S between group means at a given intensity indicates means that differ by planned comparison t tests (all $p<.05$, 2 tailed tests). The absence of an S shows that there were no differences between means.

Figure 6. Means + SEM (or Means – SEM) of ratio peak height (PH) versus intensity of stimulation for potentials evoked in PAG by ACe stimulation. Separate plots are shown for predator stressed (exposed) and control rats in the right and left hemispheres. An S between group means at a given intensity indicates means that differ by planned comparison t tests (all $p<.05$, 2 tailed tests). The absence of an S shows that there were no differences between means.

Figure 7. Means + SEM (or Means – SEM) of ratio peak height (PH) versus intensity of stimulation for potentials evoked in PAG by BLA stimulation. Separate plots are shown for predator stressed (exposed) and control rats in the right and left hemispheres. An S between group means at a given intensity indicates means that differ by planned comparison t tests (all $p<.05$, 2 tailed tests). The absence of an S shows that there were no differences between means.

Figure 8. Means + SEM of peak height of pulse 2 divided by peak height of pulse 1 (P2/P1) for the VAB – BLA pathway are graphed. Means collapsed across interpulse interval (IPI) are shown for predator stressed (exposed) and control rats for Day 1 and Day 9 studies both in right and left hemispheres. Mean + SEM for each IPI collapsed across groups and hemisphere appear to the right of group and hemisphere means. Means marked with the same letter do not differ but differ

from those with different letters (all $p<.05$, Bonferroni protected 2 tailed t tests). Means with two letters fall between means with one of the letters. An asterisk indicates that the 20 and 30 msec IPI differs.

Figure 9. Means + SEM of peak height of pulse 2 divided by peak height of pulse 1 (P2/P1) for the left BLA-PAG pathway are graphed. Means for each interpulse interval (IPI) are shown for predator stressed (exposed) and control rats for Day 9. Means marked with the same letter do not differ but differ from those with different letters (all $p<.05$, Bonferroni protected 2 tailed t tests).

Figure 10. Bmax + SEM estimated from fits of logistical dose response curves for VAB-BLA from Day 1 and Day 9 are graphed. Bmax values are graphed separately for Group, Hemisphere and Day (Day 1 or Day 9). An asterisk over the control indicates that the control group differs from the predator stressed group.

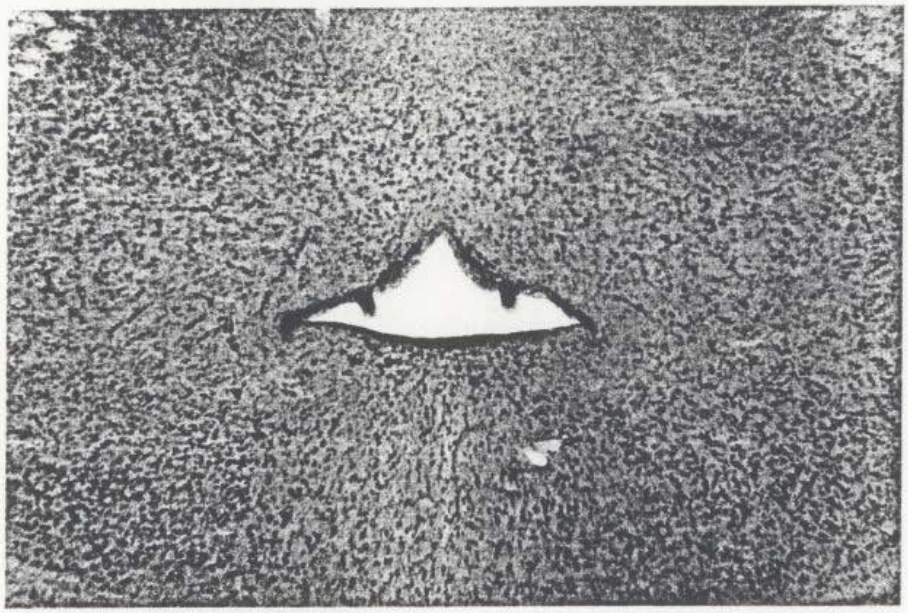
Figure 11. Bmax + SEM estimated from fits of logistical dose response curves for ACe-PAG from Day 1 are graphed. An asterisk over the control indicates that the control group differs from the predator stressed group.

Figure 12. Results from the elevated plus maze are graphed for control and predator stressed (exposed) rats. An asterisk indicates that the means differ. UPHD – unprotected head dips; UPSA – unprotected stretch attend.

Figure 13. Results from the light/dark box are graphed in this figure for control and predator stressed (exposed) rats. An asterisk indicates that the means differ.

Figure 14. Mean + SEM startle amplitude is graphed for control and predator stressed (exposed) rats with bilateral electrodes in VAB-BLA or BLA-PAG pathways. An asterisk above control means indicates a difference from predator stressed group.

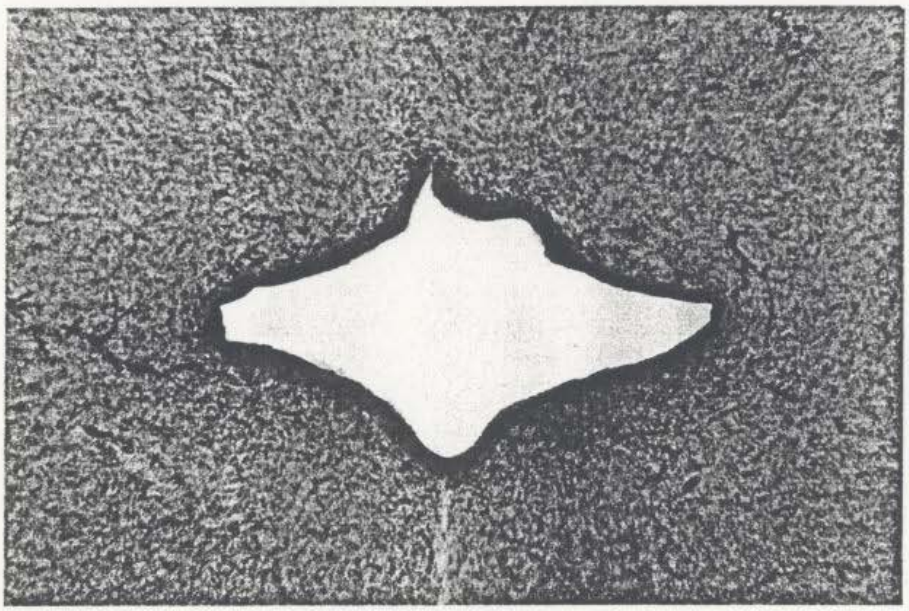
Predator Stressed



Left Hemisphere

Right Hemisphere

Handled Control



0.5 mm

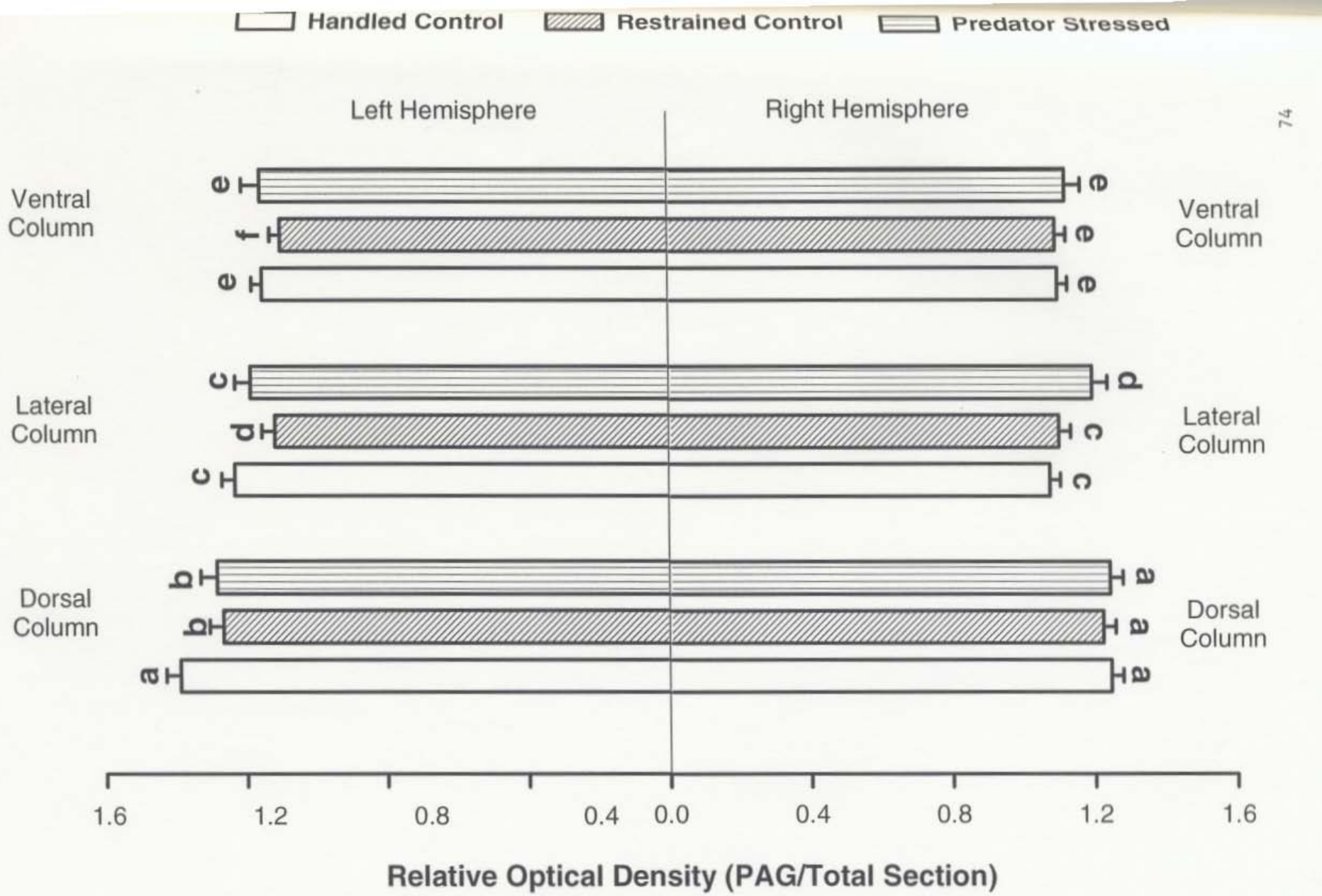

Handled Control
Restrained Control
Predator Stressed
Left Hemisphere
Right Hemisphere
Ventral Column
Lateral Column
Dorsal Column
e
f
e
e
e
e
c
d
c
d
c
c
b
b
a
a
a
a
1.6
1.2
0.8
0.4
0.0
0.4
0.8
1.2
1.6
Relative Optical Density (PAG/Total Section)

Figure 2

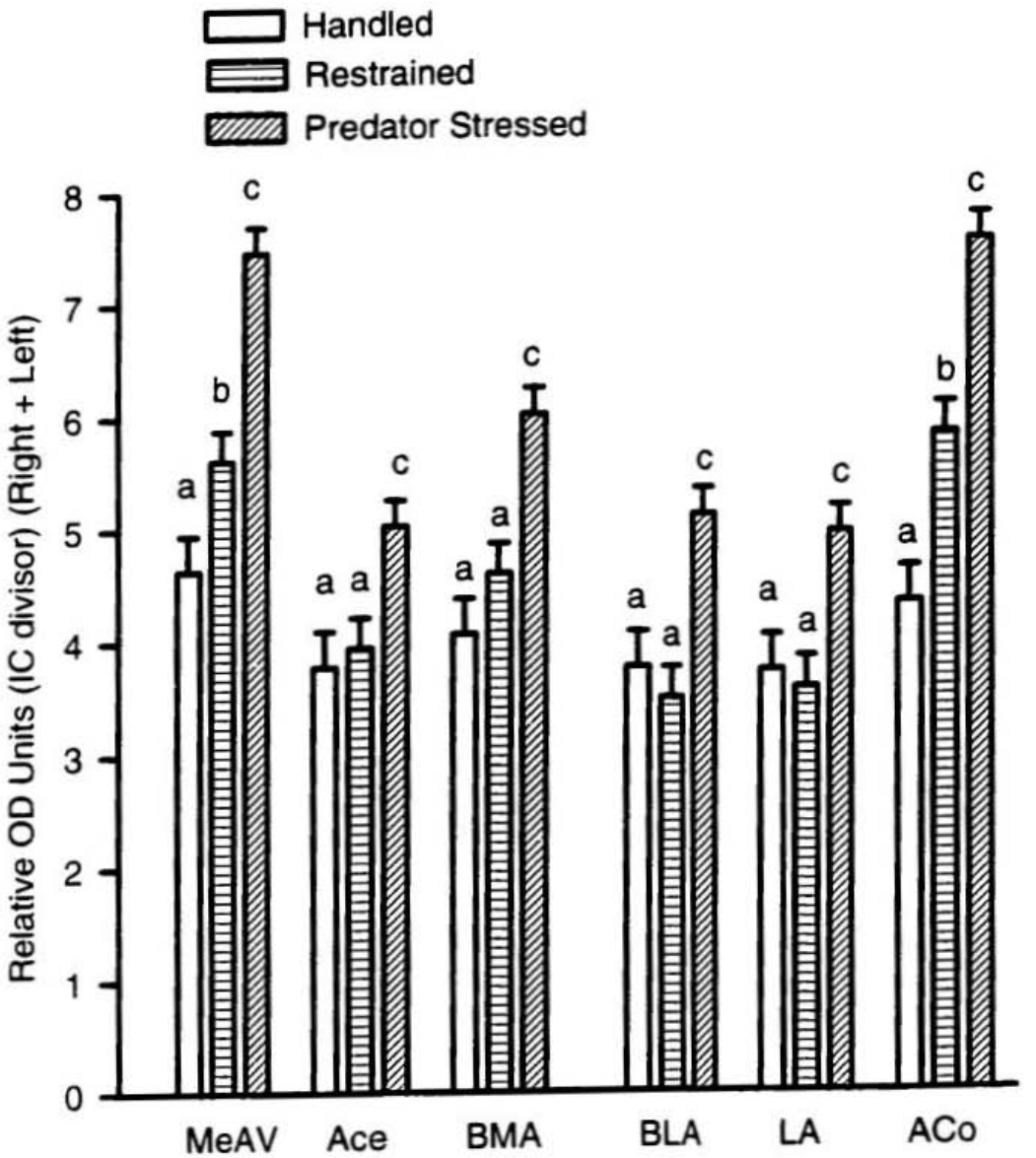

Handled
Restrained
Predator Stressed
Relative OD Units (IC divisor) (Right + Left)
0
1
2
3
4
5
6
7
8
a
b
c
a a
c
a
a
c
a
a
c
a
a
c
a
b
c
MeAV
Ace
BMA
BLA
LA
ACo

**Figure 3**

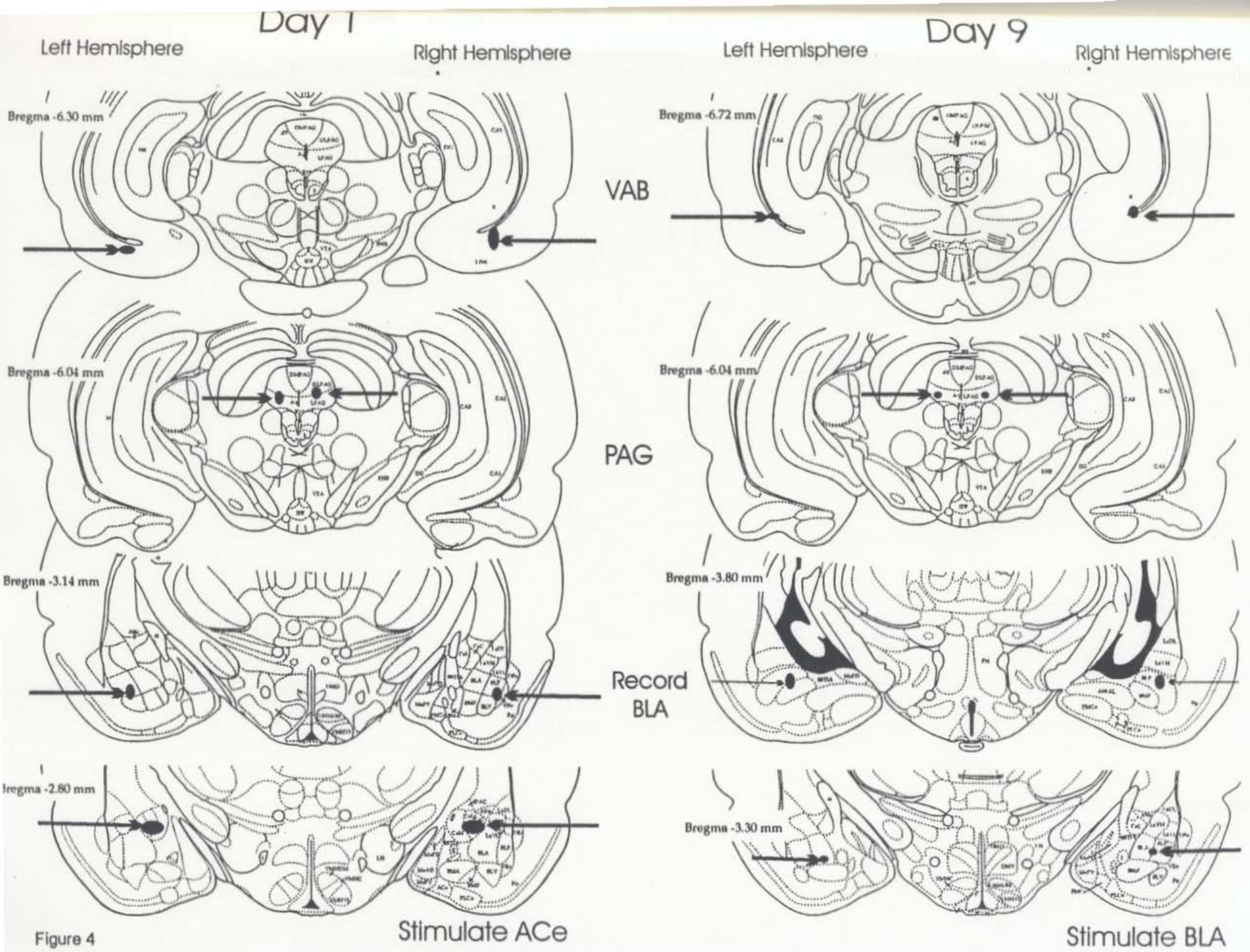

Day 1
Left Hemisphere
Right Hemisphere
Bregma -6.30 mm
Bregma -6.04 mm
Bregma -3.14 mm
Bregma -2.80 mm
Stimulate ACe
Day 9
Left Hemisphere
Right Hemisphere
Bregma -6.72 mm
Bregma -6.04 mm
Bregma -3.80 mm
Bregma -3.30 mm
Stimulate BLA
VAB
PAG
Record
BLA

Figure 4

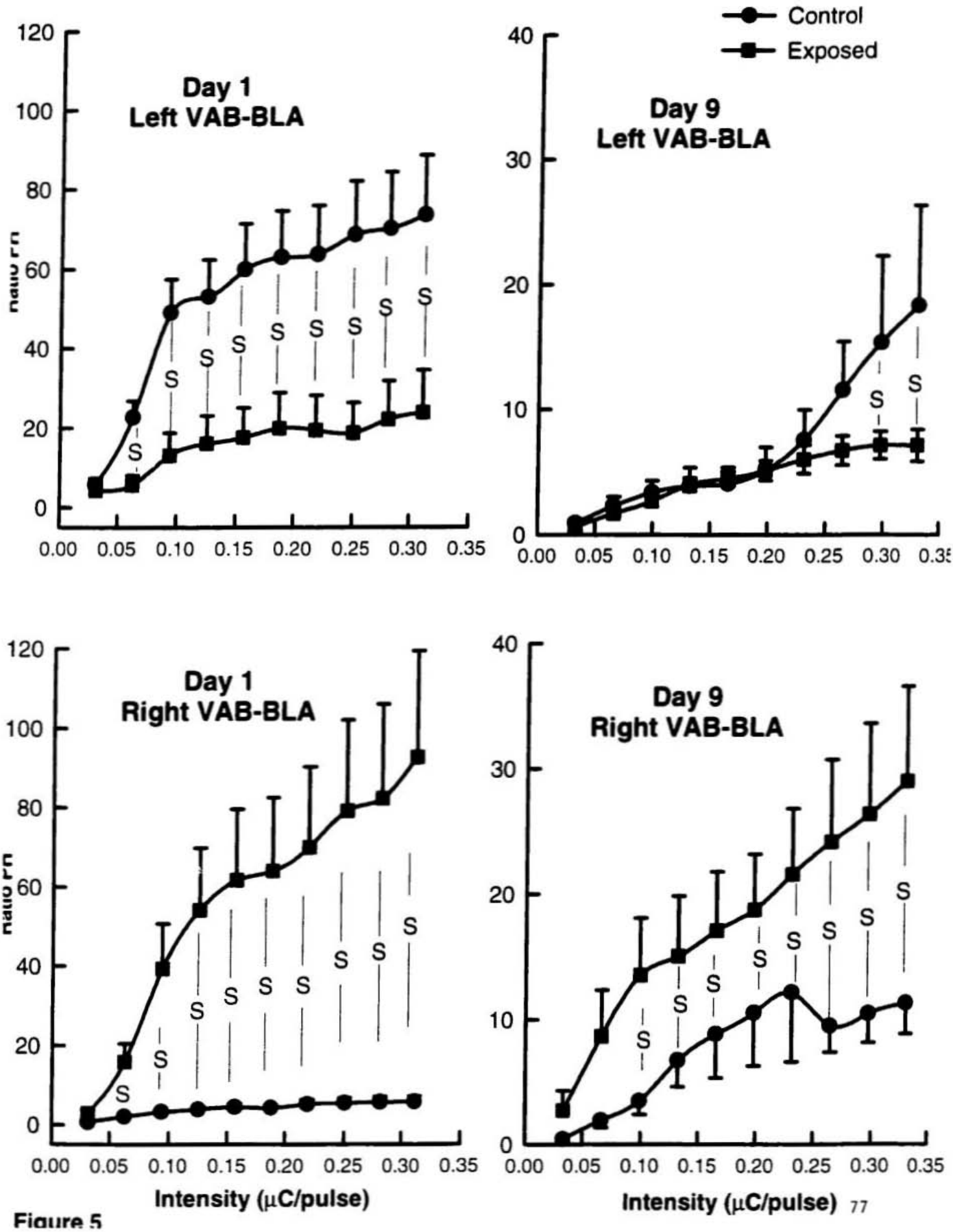

Control
Exposed
Day 1
Left VAB-BLA
Day 9
Left VAB-BLA
Day 1
Right VAB-BLA
Day 9
Right VAB-BLA
Intensity (μC/pulse)
Intensity (μC/pulse)

Figure 5

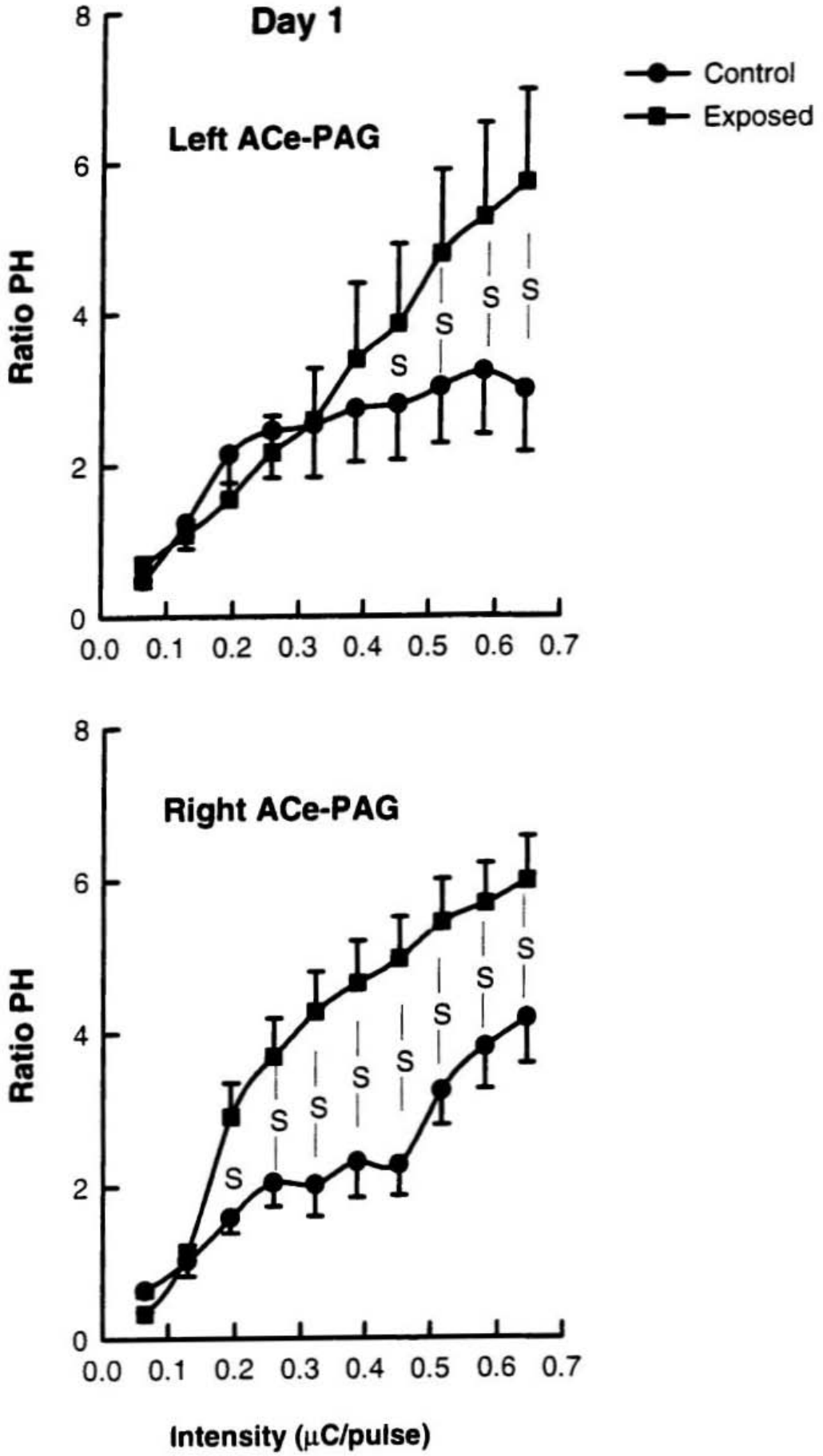

Day 1
Control
Exposed
Left ACe-PAG
Right ACe-PAG
Ratio PH
Intensity (μC/pulse)
0
2
4
6
8
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
S

Figure 6

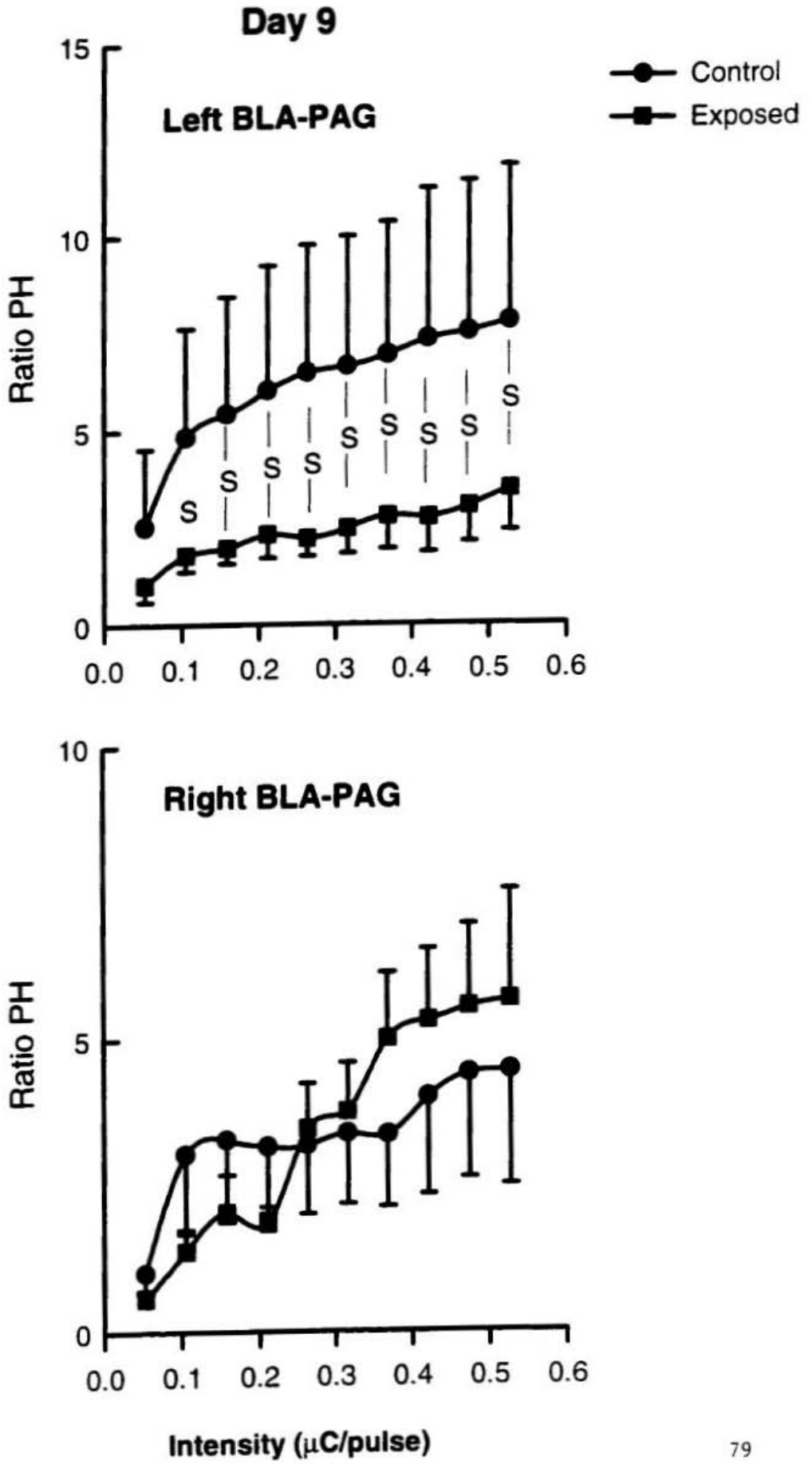

Day 9
Control
Exposed
Left BLA-PAG
Ratio PH
15
10
5
0
S
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Right BLA-PAG
Ratio PH
10
5
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Intensity (μC/pulse)

Figure 7

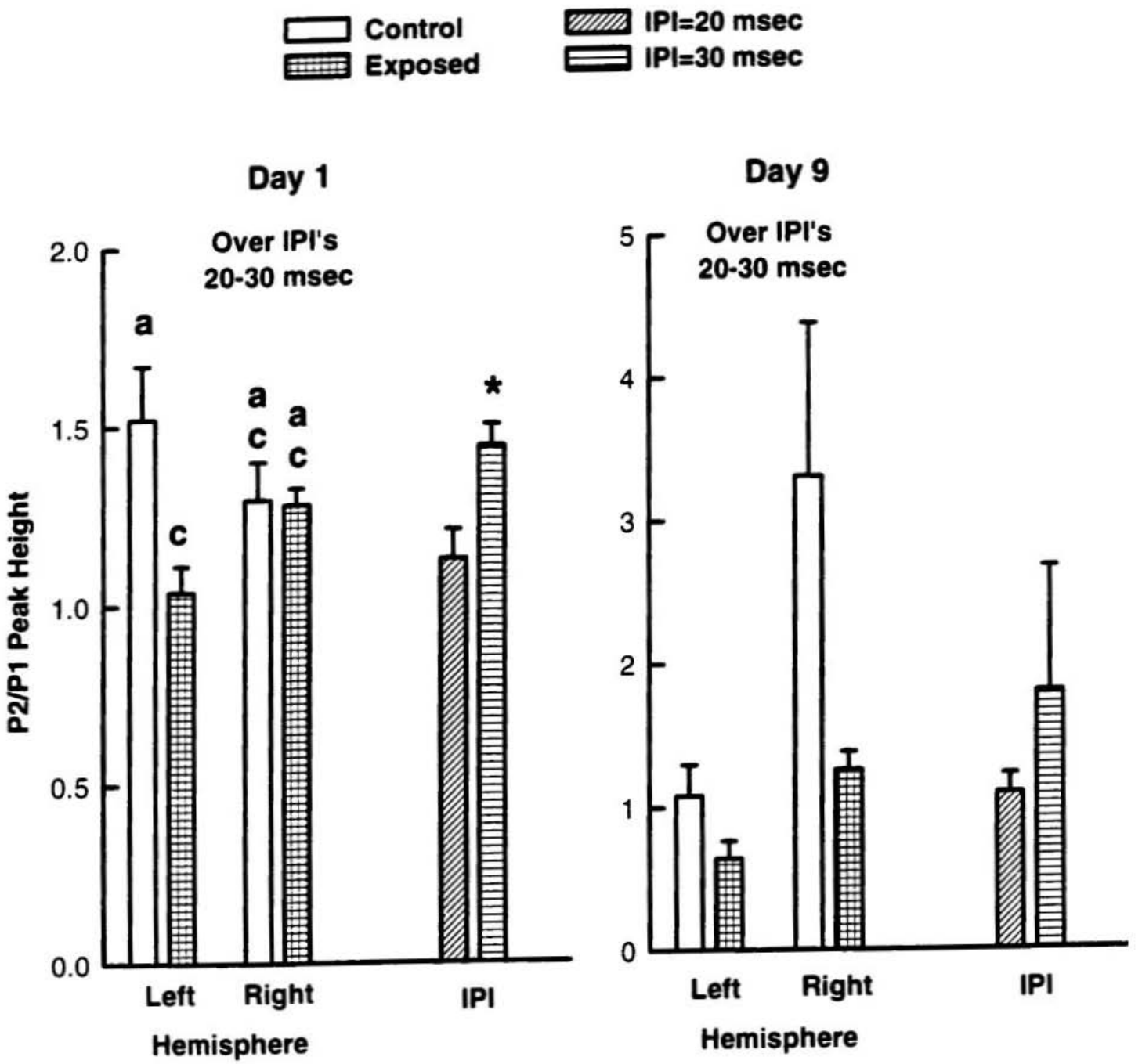

Control
Exposed
IPI=20 msec
IPI=30 msec
Day 1
Over IPI's
20-30 msec
P2/P1 Peak Height
2.0
1.5
1.0
0.5
0.0
a
c
a
c
a
c
*
Left
Right
IPI
Hemisphere
Day 9
Over IPI's
20-30 msec
5
4
3
2
1
0
Left
Right
IPI
Hemisphere

Figure 8

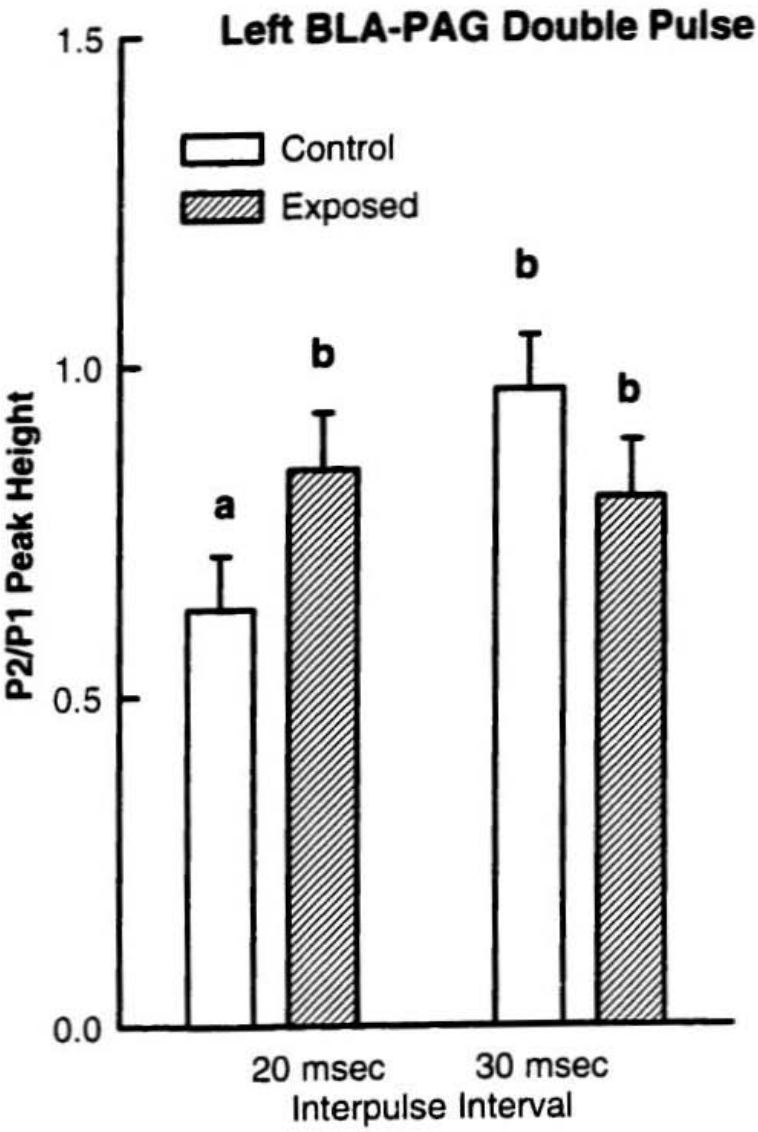

Left BLA-PAG Double Pulse
Control
Exposed
P2/P1 Peak Height
1.5
1.0
0.5
0.0
a
b
b
b
20 msec
30 msec
Interpulse Interval

Figure 9

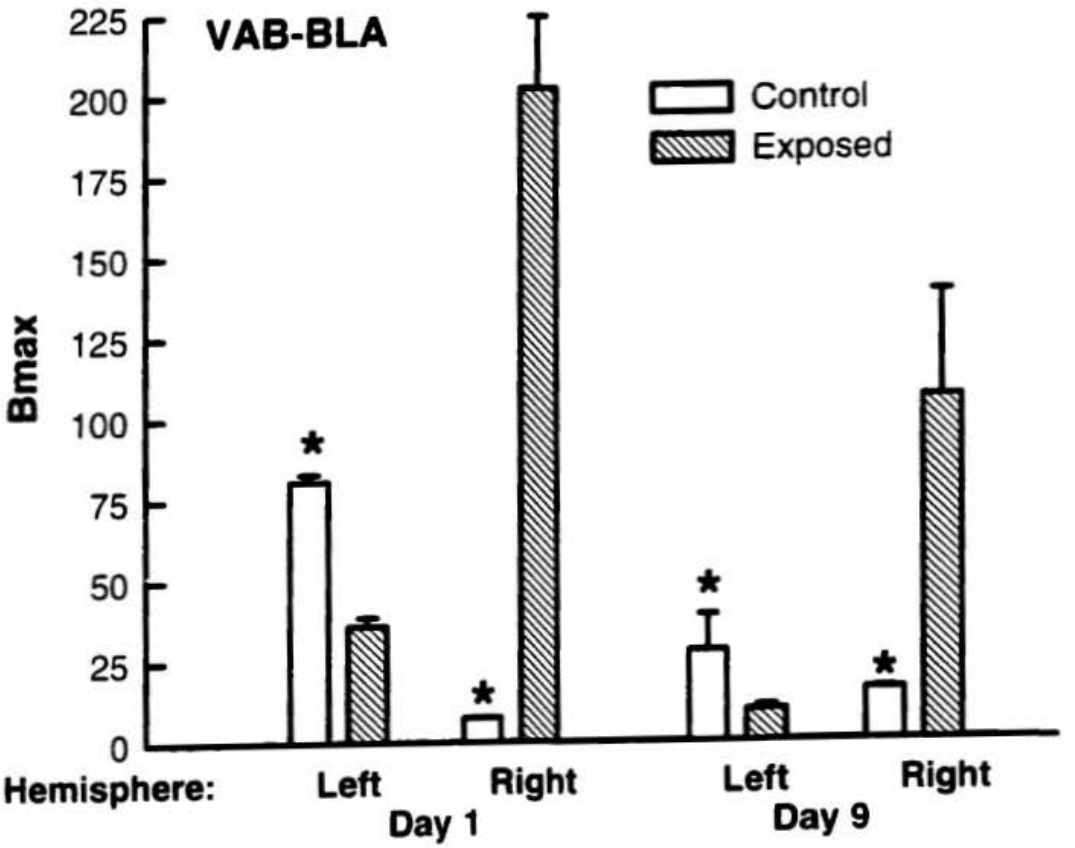

VAB-BLA
Control
Exposed
Bmax
225
200
175
150
125
100
75
50
25
0
Hemisphere:
Left
Right
Left
Right
Day 1
Day 9

Figure 10

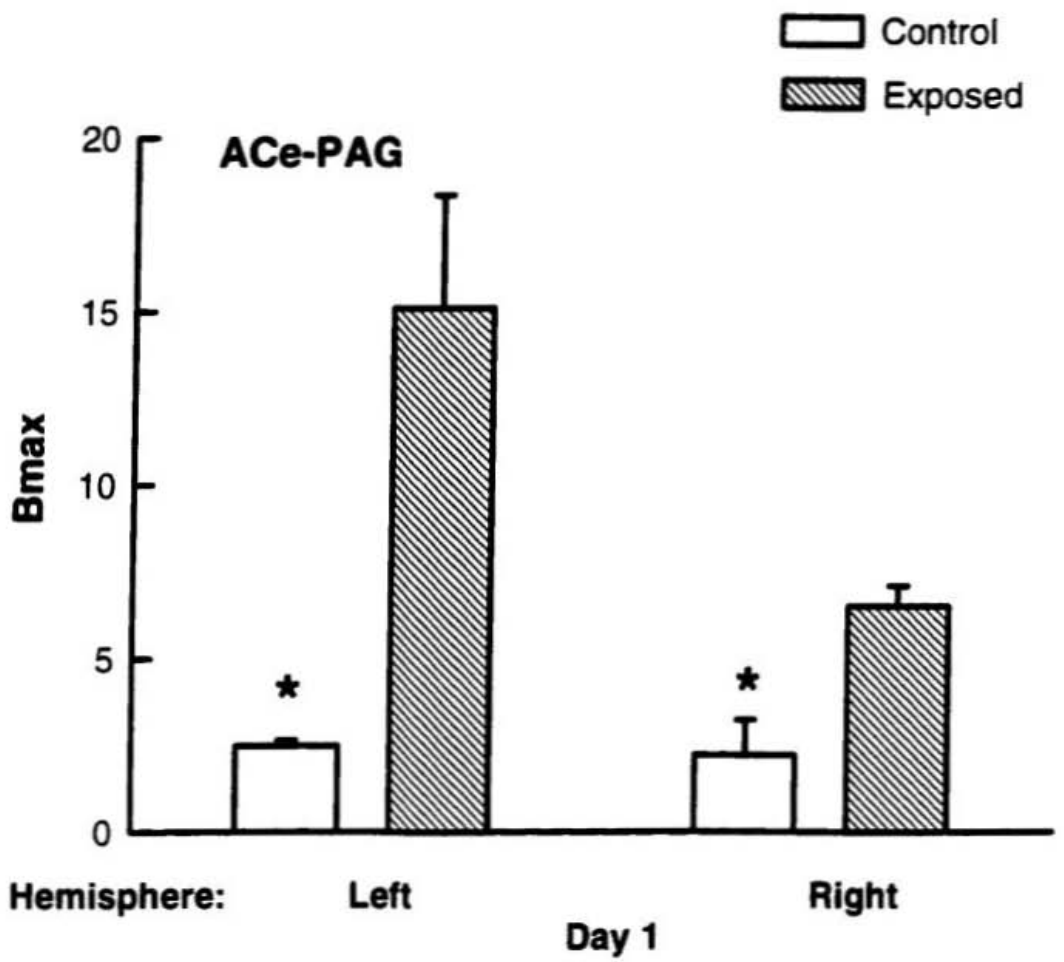

Control
Exposed
ACe-PAG
20
15
10
5
0
Bmax
*
*
Hemisphere:
Left
Right
Day 1

Figure 11

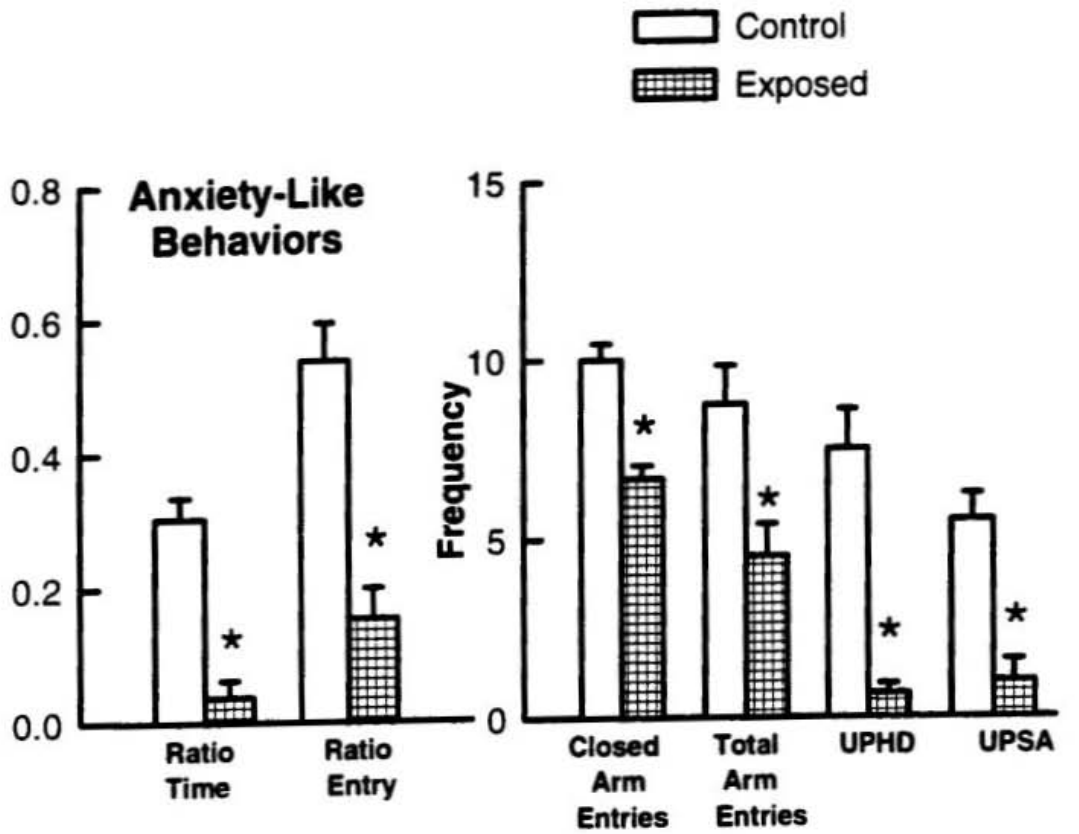

Control
Exposed
Anxiety-Like Behaviors
0.8
0.6
0.4
0.2
0.0
Ratio Time
Ratio Entry
15
10
5
0
Frequency
Closed Arm Entries
Total Arm Entries
UPHD
UPSA

Figure 12

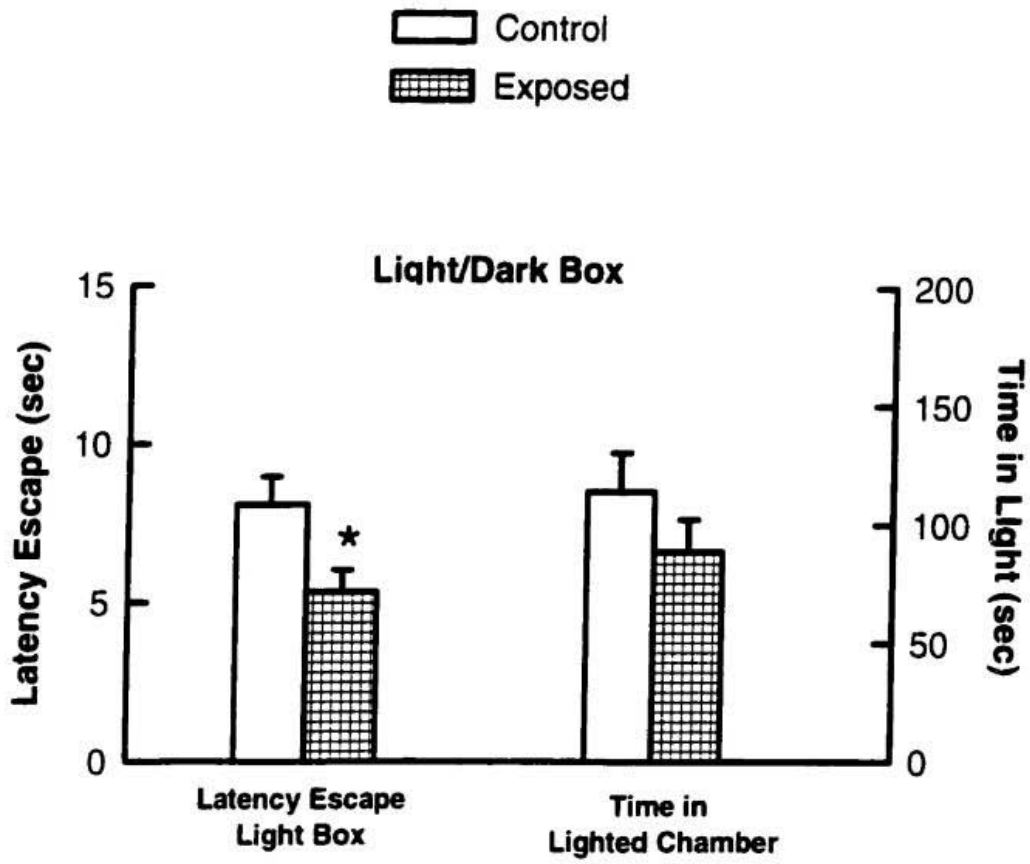

Control
Exposed
Light/Dark Box
Latency Escape (sec)
15
10
5
0
200
150
100
50
0
Time in Light (sec)
*
Latency Escape
Light Box
Time in
Lighted Chamber

Figure 13

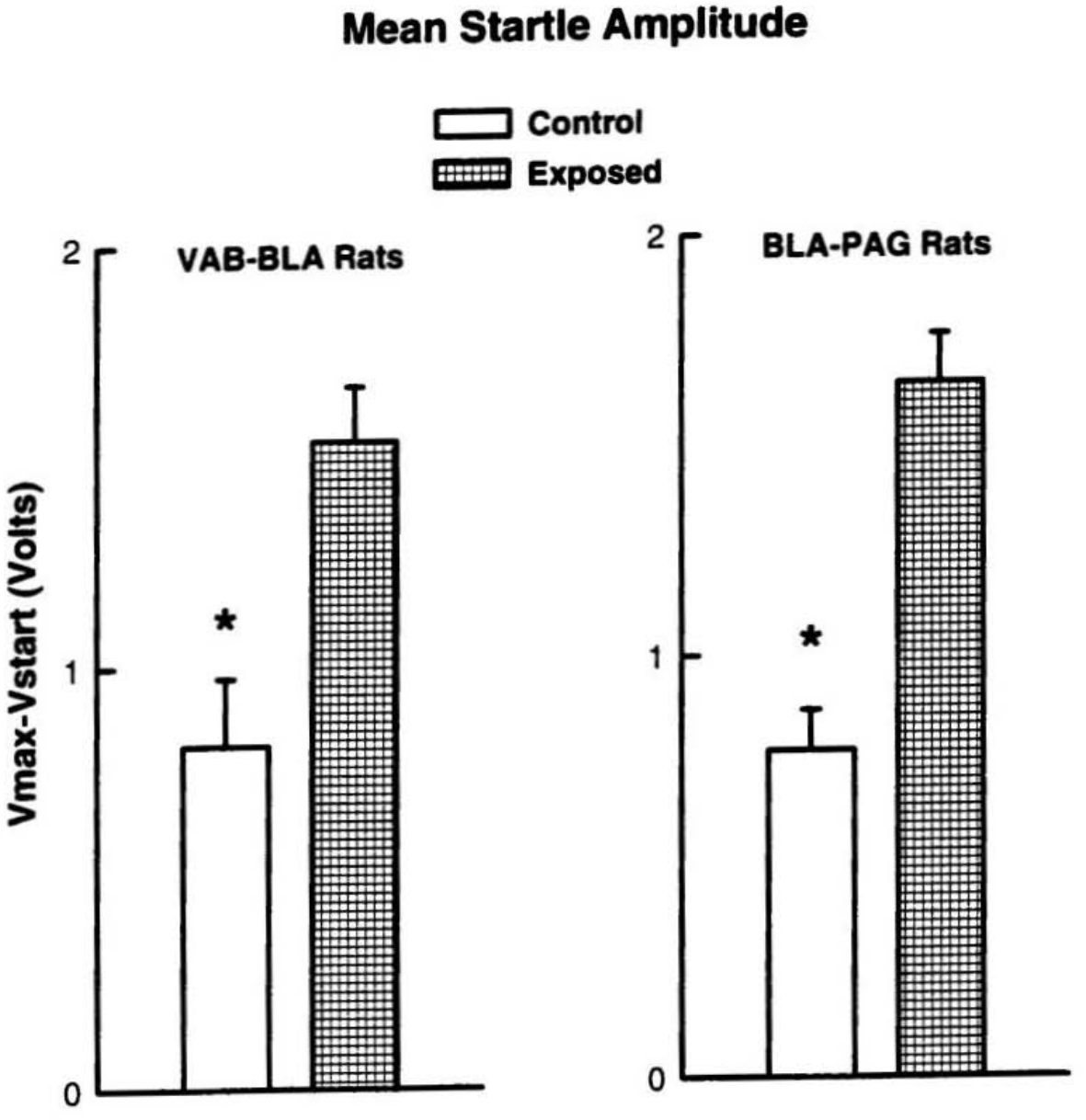

Mean Startle Amplitude
Control
Exposed
VAB-BLA Rats
BLA-PAG Rats
Vmax-Vstart (Volts)
2
1
0
*
*

Figure 14

Appendix 1.

# PROTOCOL IMMUNOCYTOCHEMISTRY

## PCREB - wells

1. Wash sections 3 times 10 minutes (can be left up to 45 minutes) with PBS. ~ 1 ml of PBS per well.
2. Blot by rolling tube on its side on a kimwipe.
3. 1 mL of solution per well (solution - 10% normal goat serum + 0.1% Triton X-100 in PBS). Cover container with plastic lid and place on a rocker for **1 hour**.
4. Wash 3 X's 10 minutes with PBS. ~1mL per well.
5. Blot as in Step 2.
6. Have the antibodies diluted and ready to use. Dilute the primary antibody in a solution of PBS, containing 2% NGS + 0.1% Triton X-100 (swirl, do not flick!).
7. 1 mL of solution into each well (may use as little as 0.8 mL per well) and incubate for **40 - 48 hours** in fridge (-4°C). Cover wells with parafilm and plastic lid to prevent sections from drying.
8. Wash 3 X's 10 minutes with PBS.
9. Blot as in Step 2.
10. Prepare the secondary biotinylated antibody (goat anti-rabbit Ab). Use the same diluent as for the primary antibody. For 10ml of buffer use 50 ul of secondary antibody (swirl). Add 1 mL of solution to each well. Cover with plastic lid and agitate for **1 hour**.
11. At this point prepare the ABC reagent as the Vector Kit instructs. To 5 ml of PBS add 50 ul (1 drop) of reagent A and mix. Then add 50 ul of reagent B, mix well.
    *** This solution must be prepared at least 30 minutes prior to use.**
12. Wash 3 X's 10 minutes 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 (cover with plastic lid).
15. Wash 3 X's 10 minutes with PBS.

16. Blot as in Step 2.
17. Make up DAB solution just prior to use (do not want the DAB in solution to turn brown) – use gloves:
    (a) Take 10 mg DAB tablet
    (b) Add DAB to 10 ml of PBS, vortex until dissolved
    (c) While swirling DAB add slowly 10 ml of PBS.
    (d) Just prior to adding DAB to sections add 60 ul of $H_2O_2$ to solution (0.1mL $H_2O_2$(30%) in 0.9mL PBS).
    (e) Add at least 1 mL (as much as 1.5 mL) to each well. Incubate (on agitator) for 5-25 minutes, monitoring for stain as they incubate. (Do not use the container as used in previous incubation).
18. Stop staining by adding PBS 3 X's 10 minutes. ~ 1mL in each slide well.

**DAB MUST BE DEACTIVATED BEFORE DISCARDING. ADD JAVEX TO DAB WHEN DAB HAS BEEN REMOVED FROM SECTIONS.**

19. Leave in PBS overnight and cover with parafilm and plastic lid.
20. Mount sections on slides.
21. Dehydration of sections.
    1. dH20
    2. 50% EtOH
    3. 75% EtOH
    4. 90% EtOH
    5. 90% EtOH
    6. 100% EtOH
    7. 100% EtOH
    8. Xylene
    9. Xylene
22. Coverslip

## Appendix 2.

### Amygdala Templates

| Templates | Range |
|---|---|
| -2.12 | (-2.12 to -2.21) |
| -2.3 | (-2.22 to -2.46) |
| -2.56 | (-2.47 to -2.66) |

### Amygdala Nuclei Template Range

| | Template | | | | | |
|---|---|---|---|---|---|---|
| | -2.12 | | -2.3 | | -2.56 | |
| Nucleus | Lateral | Vertical | Lateral | Vertical | Lateral | Vertical |
| MEAV/D | 2.8 - 3.3 | 9.2 - 8.2 | 2.8 - 3.3 | 9.4 - 8.1 | 2.7 - 3.5 | 9.7 - 8.0 |
| CN | 3.4 - 4.5 | 8.4 - 7.4 | 3.4 - 4.5 | 8.5 - 7.4 | 3.6 - 4.6 | 8.4 - 7.4 |
| BMA | 3.5 - 4.6 | 9.1 - 8.7 | 3.3 - 4.3 | 9.4 - 8.8 | 3.6 - 4.4 | 9.4 - 8.7 |
| BLA | 4.6 - 5.3 | 8.7 - 7.7 | 4.5 - 5.3 | 9.0 - 7.7 | 4.4 - 5.4 | 9.0 - 7.8 |
| LA | 4.8 - 5.4 | 7.7 - 7.3 | 4.9 - 5.4 | 7.7 - 7.3 | 4.8 - 5.4 | 7.8 - 7.0 |
| Aco | 3.2 - 4.5 | 9.8 - 9.4 | 3.1 - 4.1 | 9.8 - 9.3 | 3.0 - 4.0 | 9.9 - 9.4 |
| Internal Capsule | 3.3 - 3.6 | 6.5 - 7.0 | 3.4 - 3.8 | 6.5 - 7.0 | 6.5 - 7.0 | 3.6 - 3.8 |