POST TRAUMATIC STRESS DISORDER (PTSD) AND THE SUBSTRATES OF ANXIETY ENHANCEMENT IN THE ADULT RAT

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by

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Abstract

This study was designed to investigate the effects of the benzodiazepine receptor antagonist, Flumazenil, on an ecologically sound model of post traumatic stress disorder (PTSD) (Experiment 1). In addition, the study examined the role of N-methyl D-aspartate (NMDA) receptor physiology in the genesis of anxiety-like behavior (ALB) (Experiment 2).

Experiment 1 was designed to evaluate the effects of cat exposure and treatment with Flumazenil on anxiety and startle amplitude of Long-Evans rats. Animals were exposed to a cat for 5 minutes and then tested in the elevated plus maze 1 week later. Animals were injected with Flumazenil or sterile vehicle 10 minutes before behavioral testing. The following day startle amplitude was measured in the acoustic startle chamber. Animals given Flumazenil exhibited more head dips than vehicle injected controls. Furthermore, animals that were exposed to a cat exhibited more head dips than animals which were not exposed. In the elevated plus maze animals that were cat exposed showed significantly more anxiety-like behavior than animals that were not exposed to a cat. Flumazenil was behaviorally neutral in the elevated plus maze and had no effects on ALB of cat exposed rats. Moreover, the exposed animals showed a slower rate of habituation to the startle stimulus than animals that were non-exposed. The startle amplitudes of animals that were cat exposed or given Flumazenil were greater than for

animals given vehicle and not exposed in the first 7 blocks of startle trials. However, by the end of the eighth block, all groups had reached an equivalent startle amplitude end point.

Experiment 2 was designed to evaluate the neuropharmacology of stress induced increases in anxiety. Specifically, the role of NMDA receptors in the pathophysiology of anxiety were investigated. Animals were cannulated in the basolateral amygdala in the left or right hemisphere or bilaterally. In other animals cannulas were implanted, and they were then handled but not exposed to a cat or intracranial injection. Experimental animals were injected with either the NMDA receptor antagonist, MK-801, or sterile saline, as appropriate, 30 minutes prior to exposure to the cat. It was found that the operated controls spent significantly more time in the open arms of the plus maze than either vehicle or MK-801 groups, which did not differ from each other. Thus, cat exposure increased plus maze anxiety(decreased open arm exploration) one week after the exposure equally in both the vehicle and MK-801 groups. However, MK-801 partially blocked the effects of cat exposure on Risk Assessment when injected into the left amygdala or bilaterally. Finally, MK-801 into the right and left hemispheres reduced the magnitude of the startle amplitude to the level of an operated-handled non-exposed control. The implications for anxiety research and PTSD are discussed.

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Table of Contents

List of Figures

Figure 4. Mean ± SEM of Relative Frequency Risk is plotted for the three Drug conditions. Plotted separately are the Drug conditions collapsed over all cannula placements (Over All), and separately for rats with cannulas in the right (Right), left (Left) and both (Bilateral) amygdalas. For each set of three means, bars marked differently differed significantly from one another, while bars marked similarly did not differ from each other. Means having two marks fell between other means possessing these marks.

Figure 5. Plotted in the top panel are mean startle amplitude (Vmax-Vstart) for the Right Amygdala Exposed animals (Saline + MK-801) in the 1st day of startle (80 trial). Data are presented over the 16 blocks of 5 trials/block. Plotted in the middle panal are mean startle amplitude (Vmax-Vstart) for the remaining groups on the 1st day of startle, (operated-handled animals, RBH, LBH, BBH. There are no trial effects across the 16 blocks. Plotted in the bottom panel is the mean startle amplitude (Vmax-Vstart) for the Handled only animals on the 1st day of startle. The data show a wide fluctuation of startle amplitude over the 16 blocks.

Figure 6. Panel illustrates the startle amplitude data for the Handled only animals. The curve represents an exponential fit to smoothed (15%) data (DF adj =0.679). Plotted values are the average startle amplitude (Vmax-Vstart) for the group over 16 blocks. Blocks consisted of 5 acoustic bursts.

Figure 7. Top panel illustrates mean startle amplitude collapsed across 80 trials for the operated animals in the 1st day of startle Experiment 2. Data are presented by hemisphere and by group. The handled animals represent the mean startle amplitude for RBH (operated in the right hemisphere and handled), LBH (operated in the left hemisphere and handled) and BBH (operated bilaterally and handled). The saline + Exposure animals represent the mean startle amplitude for RBS (operated in the right hemisphere, given saline and exposed), LBS (operated in the left hemisphere, given saline and exposed) and BBS (operated bilaterally, given saline and exposed). The MK-801 +Exposure animals represent the mean startle amplitude for RBM (operated in the right hemisphere, given MK-801 and exposed), LBM (operated in the left hemisphere,given MK-801 and exposed) and BBM (operated bilaterally given MK-801 and exposed). Differences between groups are indicated with a" @ "sign. The bottom panel illustrates the time to reach maximum startle amplitude. Data are presented as in the top panel. There were no differences between any of the groups in either of the hemispheres or in those animals implanted bilaterally.

Figure 8. Top panel illustrates mean startle amplitude collapsed across 20 trials for the 2nd day of startle in Experiment 2. Data are presented by hemisphere and by group. The handled animals represent the mean startle amplitude for RBH (operated in the right hemisphere and handled), LBH (operated in the left hemisphere and handled) and BBH (operated bilaterally and handled). The saline + exposure animals represent the mean startle amplitude for RBS (operated in the right hemisphere, given saline and exposed, LBS (operated in the left hemisphere, given saline and exposed) and BBS (operated bilaterally, given saline and exposed. The MK-801 + Exposure animals represent the mean startle amplitude for RBM (operated in the right hemisphere, given MK-801 and exposed), LBM (operated in the left hemisphere, given MK-801 and exposed) and BBM (operated bilaterally, given MK-801 and exposed). Differences between groups are indicated by a @ sign. The bottom panel illustrates the time to reach maximum startle amplitude. Data are presented as in the top panel. There were no differences between any of the groups in any of the hemispheres or in animals implanted bilaterally.

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. Of the class of benzodiazepine drugs, there are various types with different actions. There are agonists, such as valium which are anxiolytic. There are inverse agonists, such as FG-7142, which induce anxiety. Finally, there are antagonists of the receptor such as Flumazenil (R0 15-1788). Antagonists, in general, are able to reverse the effects of both agonists and inverse agonists without altering mood or behavior (File and Baldwin, 1989). Flumazenil, however, is a very interesting compound in that its effects on mood and behavior appear to depend on the dose that is administered as well as the situation in which it is given.

Applications of these compounds have produced marked improvement in patients suffering from anxiety based disorders. One such disorder that has not been so easy to treat is Post Traumatic Stress Disorder (PTSD). As recently as 1989 there have been no trials of drug treatment for these patients, with the major strategy being psychotherapy (Tyrer, 1989). Given this fact, our laboratory has developed an animal model of PTSD with good ecological validity. To further validate this model we have decided to test the benzodiazepine antagonist, Flumazenil. Flumazenil has been shown to provoke panic attacks in panic disorder patients (Randall, Bremner, Krystal, Nagy,

Heninger, Nicolaou and Charney, 1995). Conversely, Flumazenil has been shown to be behaviorally neutral in patients with PTSD. It appears that Flumazenil affects varying types of anxiety in different ways. If these actions are replicated in our model of PTSD it will add to the ecological validity of the model and provide a tool to investigate novel therapies. In addition, this thesis was designed to examine the neuropharmacology of anxiety that is associated with our model.

The following sections will review the research on Flumazenil in studies involving humans and animals. In addition, several animal models of PTSD will be introduced. These models will be discussed in the context of whether they are suitable models of this disorder. The next series of sections will examine a model of PTSD that was developed in this laboratory. Progress made in understanding the functional neuroanatomy and neuropharmacology of anxiety produced by traumatic stress will be reviewed.

Human Research on Flumazenil

Work in the 1980's examined effects of Flumazenil on psychopsysiological performance and subjective experience of normal human subjects. One such study by Higgitt, Lader and Fonagy (1986) noted that after 100 mg of Flumazenil (oral), both the Delta and the Theta waves of the EEG were reduced. There was also a significant drop in the systolic blood pressure

of the subjects that peaked about 2 hours after the dose was ingested. In addition, Flumazenil significantly slowed the subjects' time to react to auditory stimuli. Of further interest, subjects who ingested the lower dose (30 mg) experienced a significant increase in contentedness (Higgit et al. 1986).

Other studies have shown several different actions of Flumazenil depending on the state of the patient. One study showed Flumazenil with partial agonist properties in reducing deceleration of eye movements in both control and panic patients. Conversely, in control patients, Flumazenil produced no subjective effects, yet produced anxiety in panic patients (Wilson, Glue and Nutt. 1992). Nutt (1995) in a study involving 6 PTSD patients and 6 healthly controls found that 2 mg of Flumazenil did not provoke panic attacks or any other PTSD symptom in either the experimental group or the control group. These data are very interesting from a pharmacological viewpoint. It suggests that PTSD and panic disorder are governed by a differing receptor physiology.

Kapczinski, Curran, Gray and Lader (1994) studied the effects of Flumazenil on the anxiety level of the subjects participating in a public speaking test. Public speaking increased anxiety as was expected, but Flumazenil (1 mg i.v) blocked this increase. Moreover, Flumazenil had the most profound effect on the anticipatory anxiety of having to give the talk. The authors suggested that the effects of Flumazenil may be mediated via an

antagonism of some endogenous inverse agonist which was produced by the stress. Since Flumazenil was most effective in attenuating anticipatory anxiety it was suggested that benzodiazepine receptors were acting as a triggering system for anxiety situations (Kapczinski et al, 1994).

The authors speculated that the set-point in this receptor system could be shifted to an agonist or antagonist conformational state depending on the stresses of the environment They speculated that Flumazenil would shift the conformation towards a neutral point. Thus, Flumazenil could act as either an agonist, antagonist or inverse agonist depending on the set point of the system (Kapczinski et al, 1994). This idea is relevant to the present study in testing a compound such as Flumazenil on generalized anxiety associated with PTSD.

Of relevance to this theory are the findings of Randall and coworkers (1995), they examined a group of 14 Vietnam combat veterans. In this study, it was observed that a 90 second intravenous infusion of 2 mg of Flumazenil did not produce any increases in anxiety or PTSD symptomatology in patients with PTSD. Conversely, Flumazenil was panicogenic in patients who suffered from panic attacks. It was suggested by the authors that the benzodiazepine complex is in a different state in the two disorders.

Overall Flumazenil appears to be behaviorally neutral in patients who suffer from PTSD. It has also been found to be behaviorally neutral in normal

subjects. Research in animals has provided additional insights into the neurophysiology of the actions of Flumazenil.

Animal Research on Flumazenil

As in human research, dose of Flumazenil as well as the behavioral state of the animal are factors in how animals respond to the drug. Early work demonstrated that Flumazenil in dose ranges of 4 to 10 mg/kg in rats reduced social interaction. This anxiogenic effect was produced without affecting motor activity. In contrast, when the dose was increased to 20 mg/kg there was a marked increase in the level of social interaction, an anxiolytic effect (File et al, 1982).

In contrast, Flumazenil (10-20 mg/kg) is without anxiogenic activity in the elevated plus maze, another test of rodent anxiety (Pellow and File, 1986). This observation parallels the human findings that Flumazenil is behaviorally neutral in normal persons. It was further hypothesized by the authors that the actions of benzodiazepine antagonists may depend on both the test situation, and more importantly, the baseline level of anxiety at the time of administration (Pellow and File, 1986).

Work in our laboratory, in the cat, is consistent with this view. Flumazenil has been shown to be behaviorally neutral in naive cats. However, under certain circumstances Flumazenil is anxiolytic. It reverses

defensiveness of cats towards rodents, but only in cats whose basal defensiveness was raised lastingly by the compound FG-7142 (Adamec, 1994). It is of further interest that FG-7142 mimics many of the effects of exogenous stressors (Biggio et al., 1987; Mcgregor & Atrens., 1990).

Other studies have attempted to look at the pharmacokinetics of Flumazenil in the rodent as well as the human subject. The half life of Flumazenil in rat brain was determined to be about 16 minutes. However, in human plasma it is less than 30 minutes (Lister et al, 1984). Given this quick elimination time it becomes very surprising that Flumazenil can reverse benzodiazepine effects for up to 6 hours after it is administered (Lister et al, 1984).

File and Hitchcott (1990) present an interesting theory of benzodiazepine dependence which could be of use in attempting to understand the actions of Flumazenil in these models. Accordingly, the critical factor is the anxiety level of the animal prior to drug administration. When the animals are anxious, Flumazenil has an anxiolytic effect, conversely, if the animals are not anxious, Flumazenil exerts an anxiogenic effect. The main tenet of this theory is that Flumazenil has the ability to reset the benzodiazepine receptor back to a baseline state (File and Hitchcott, 1990).

The authors further postulate that the effects of Flumazenil could be via an effect on an agonist or an inverse agonist which is released due to the

stressful situation (File and Hitchcott, 1990). This idea agrees with Adamec (1990) who has suggested that under normal conditions Flumazenil functions as a behaviorally inert antagonist. However, during stress, there is either an increase in the production of an endogenous anxiogenic ligand, or a reduction of an endogenous anxiolytic ligand. These explanations allow for a possible understanding of the bidirectional effects of Flumazenil.

Since Flumazenil is behaviorally neutral in human patients with PTSD it seems logical to evaluate its role in an animal model of the disorder. If Flumazenil behaves similarly in an animal model of PTSD, it strengthens the clinical relevance of the model. The following section will review several animal models of PTSD. The section will conclude with an examination of an ecologically valid animal model of PTSD with good face validity that has been recently developed in this laboratory.

Models of Post Traumatic Stress Disorder (PTSD)

To be considered a good model of PTSD, an animal model of PTSD should parallel clinical features of the disorder in humans. According to the DSM-III there are six criteria of which 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 finally an exaggerated startle response. Finally, the DSM IV-R states that PTSD chronic anxiety is present if it persists for 4 months or longer.

In addition to the clinical criteria used to diagnose the disorder it would be useful if the animal model matched some biochemical abnormalities which are associated with the disorder. According to Van der Kolk (1994), PTSD patients have abnormal stress hormone release. Abnormal levels of several modulators such as norepinephrine, oxytocin, cortisol, and vasopressin have also 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).

One of the symptoms of PTSD, the exaggerated startle response, warrants further discussion in consideration of developing animal models of this disorder. Butler and colleagues (1990), compared a group of combat veterans with PTSD with a group of combat veterans without PTSD on the eyeblink reflex response. PTSD patients had a significantly greater response amplitude than the control subjects (Butler et al, 1990). Kolb (1987) found similar differences using blood pressure and galvanic skin response in response to combat sounds in PTSD patients and controls. PTSD subjects showed a greater response to the sounds than their controls.

In addition to an exaggerated startle response, PTSD patients also take

longer to habituate the acoustic startle response. For example, Orr et al (1995), using skin conductance response magnitude as a measure of startle found magnitude of the response decreased more quickly in control subjects than in the PTSD patients. It is interesting to note, however, that all subjects in the study were able to reach the skin conductance nonresponse criterion at the end of the study.

Hence, a good animal model of PTSD should be able to produce (i) a long lasting anxiety, (ii) fluctuation in the levels of stress hormones, (iii) an altered memory for the stressful event, (iv) an exaggerated acoustic startle response (ASR), and a delayed onset of habituation of the acoustic startle response.

It appears that PTSD patients suffer from generalized heightened arousal as well as physiological reactions to specific things in the environment (Pitman et al, 1993). Observations of traumatized patients has revealed that once an individual has experienced an emotion in the extreme, the individual has a heightened chance to experience it to a further extreme (Pitman et al, 1993). Similar notions of sensitization are present in animal models of PTSD.

One sensitization model of PTSD is emotive biasing. The main tenet behind this theory is that repeated stimulation of a limbic substrate which is associated with a certain emotional state eventually alters the substrate, enhancing its function (Adamec, 1978). Adamec (1991) has found that cats are

different from birth with respect to the relative strength of their defensive responses when exposed to a rodent. The neurophysiological correlate of this appears to be a strengthening of inputs from the basal amygdala to the ventromedial hypothalamus (VMH). In terms of PTSD, it is a critical piece of the puzzle as electrical stimulation of this same pathway leads to long lasting increases in defensive behavior toward rodents (Adamec, 1991),

From emotive biasing one could look at another related model, kindling. Kindling is a phenomenon where repetitive, subconvulsive electrical stimulation of limbic circuits comes to evoke convulsions (Pitman et al, 1993). In accordance with the kindling model of PTSD, the repeated experiencing of a specific trauma results in a long term sympathetic arousal which is mediated by the locus coeruleus (Van Der Kolk, 1987). However, according to Pitman and coworkers (1993), kindling is not as good a model as emotive biasing as it has an electrophysiological basis, as opposed to a sound behavioral basis. However, the effects of kindling on anxiety have important clinical implications.

Adamec (1990) examined the results of kindling on anxiety in the rat. It was found that kindling in the right amygdala increased anxiety in rats for at least one week after the last stage 5 seizure. Kindling was demonstrated to lastingly increase the excitability of amygdalar circuitry. The data from this study are also consistent with the notion that kindling heightens the

normal functioning of limbic substrates.

Another model of PTSD that has gained wide popularity is inescapable shock (IS). In this model animals are exposed to inescapable stress in the form of electric shocks from which they cannot escape. It has been noted that animals who have experienced inescapable shock later display a reduced initiation of normal behavior, an apparent cognitive deficit, and finally symptomatology associated with emotional instability (Rosen and Fields, 1988).

Van der Kolk and others (1985) consider IS an excellent model of PTSD in that it parallels both the biochemical and behavioral changes seen in PTSD. Specifically, it was noted that IS increases the releasable stores of norepinephrine as well as the production of MHPG. However, one of the shortcomings of the IS model is that it does not accurately model some of the late onset symptoms which characterize PTSD (Jones and Barlow, 1990). Another important point is that the IS model fails to account for the fact that PTSD can develop after one traumatic event as opposed to several repeated experiences (Yehuda and Antleman, 1993). Recently an animal model of PTSD with a high degree of face validity has been developed The model consists of exposing a rat to a cat for a 5 minute period (Adamec and Shallow, 1993). The exposure of the rat to a cat produces a long-lasting increase in anxiety-like behavior (ALB) lasting at least three weeks after the exposure. If one were to use a comparison ratio of life span, it has been estimated that

7.5 days of a rat's 3 year life span was equivalent to 6 months of a human living to be 72 years of age. Hence, in comparison to a human's life span, the animal would have experienced chronic anxiety for roughly 18 months in a human's life span. This time line meets the criterion as set out in the DSM-IV-R where anxiety is considered chronic if it persists for 4 months or longer. With the development of this animal model, our laboratory has been searching for the substrates of anxiety enhancement in the adult rat.

The next series of sections will detail this search for the mechanisms of anxiety enhancement. The first part of the discussion will deal with the defence/aversive system in the brain. It will be followed by a discussion of the N-methyl-D-aspartate (NMDA) receptor and its role in the neurophysiology of anxiety. Following this there will be a section on the proposed mechanisms of anxiety, the hemispheric asymmetry of the anxiety mechanisms as well as the specific location of these changes. The section will close with some conclusions and justifications for the current study.

Defence/Aversive System of the Brain

The amygdala is part of the brain's defence/aversive system. Other structures in this system are the ventromedial hypothalamus(VMH) and the dorsal periacqueductal gray (dPAG). There is a large body of evidence which suggests that fear induced by the environment is relayed and processed in a

rostral-caudal direction (Silveira et al, 1993). It has been speculated that stimuli arrive at the amygdala after cortical analysis, where they are tested for the degree of threat that they pose. From here the information is relayed to the periacqueductal gray which organizes an appropriate response (Silveira et al, 1993).

A variety of studies have examined the role of the PAG in anxiety. 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). Effects of Midazolam were antagonized by 80 nMol of Flumazenil injected into the PAG. However, Flumazenil injected into the PAG did not attenuate the anxiolytic effect of systemically injected diazepam. Therefore, the dorsal PAG is not the only brain structure involved in the anxiolytic actions of benzodiazepine agonists (Russo et al, 1993).

Other data implicate NMDA receptors in the PAG in rodent anxiety. Local block of NMDA receptors in the PAG, by the competitive antagonist AP7, dose dependently decreases plus maze anxiety (Guimaraes et al, 1991). The next section will deal with the role of NMDA in the expression of fear and anxiety as well as its role in aversive memory.

N-Methyl-D-Aspartate (NMDA): Fear, Anxiety and Aversive Memory

The N-Methyl-D-Aspartate (NMDA) receptor complex has been implicated in long term potentiation (LTP), as well as pathology associated with cerebrovascular accidents (i.e strokes) and currently with mechanisms of anxiety.

Understanding of the role of the NMDA receptor complex in the pathophysiology of anxiety has grown in recent years. Blockade of NMDA receptors in a brain area known to be involved in fear and anxiety (the amygdala) prevents the establishment of fearful memories (e.g Campeau et al., 1992; Mindy et al.,1990). In another study, Fanselow et al(1991) infused AP7 into the basolateral nucleus of the amygdala prior to a training regimen and found that it blocked conditioned freezing 24 hours later. This action of NMDA is consistent with an hypothesis, since supported in this laboratory, that lasting increases in animal anxiety are due to LTP in neural pathways involved in fear in animals, and anxiety in humans. These considerations are of further relevance to PTSD.

The associative LTP that has been linked to fear conditioning in animals is the condition whereby the activation of a weak input onto a postsynaptic cell becomes paired with activation of another input onto the same cell which is stronger than the first input. After a few pairings of these inputs, the weaker of the two becomes potentiated (Davis et al. 1994). The activation of

this weak input elicits the release of excitatory amino acids, principally glutamate. Glutamate, in turn attaches to both the NMDA and the non-NMDA receptors (AMPA,Kainate, Quisqualate) receptors on the postsynaptic cell. However, the binding of the ligand has no effect as the calcium channel in the receptor is blocked by a Mg ion when the cell is in the resting state. When the neuron becomes depolarized by a strong input, the Mg ion is displaced which allows calcium ions to rush into the cell. This calcium in the cell initiates a cascade of events which leads to a long-lasting potentiation of the initial weak input (Davis et al, 1994). It follows that, if these receptors are blocked by an antagonist of the receptor such as AP7 (competitive antagonist) or MK-801(non-competitive antagonist), then glutamate will be unable to bind and no potentiation will be elicited (Davis et al, 1994). This idea forms the basis for part of the present study.

Recently, the hypothesis that NMDA LTP mediates increased anxiety in our animal model was tested and supported. It has been found, in rats, that a systemic injection of the non-competitive antagonist MK-801 (0.30 mg/kg), 30 minutes prior to exposure to a cat blocks the initiation of anxiety-like behavior measured 1 week later in the elevated plus maze (Adamec, Shallow and Budgell,1996; Submitted to Journal of Psychopharmacology). MK-801 administered 30 minutes after the exposure to the cat was without effect, however. These data suggest that NMDA receptors are involved in the

initiation, but not the maintenance of neural activity mediating increases in anxiety following stress. The question still remains, however, as to where in the brain these changes take place. Some recent research has examined several of the different nuclei of the amygdala in terms of their role in both anxiety and aversively motivated memory.

Parent and McGaugh (1994) infused lidocaine bilaterally into both the central nucleus and the basolateral nucleus of the amygdala. The infusions occurred immediately following training on an inhibitory avoidance task. Retention was assessed 2 days later. Infusions into the central amygdala did not affect retention performance. Conversely, infusions into the basolateral amygdala significantly affected the animals retention. Interestingly, infusions given 6 hours after the training regimen affected the retention, but infusions 24 hours later had no effect on the animals retention (Parent and McGaugh, 1994).

In addition to nuclei differences in the retention of aversive memory, there are hemispheric asymmetries in role played by limbic structures in animals. This is of particular interest in the study of PTSD, as hemispheric differences have been found in patients suffering from PTSD. Rauch and colleagues (1995), using Positron Emission Tomography (PET) in PTSD patients, found increased blood flow in right-sided limbic, paralimbic and visual areas following reminders of the traumas of the patient. Interestingly,

there were concomitant decreases in regional blood flow to the inferior frontal and middle temporal cortex of the left hemisphere (Rauch et al, 1995). The results of this study suggest that emotions associated with PTSD are localized in the right hemisphere. Similar data have emerged in animal studies.

The Hemispheric Asymmetry of Anxiety: Laterality of Emotional Affect

Recent work with rodents has demonstrated that the left and right amygdala play different roles in the acquisition and expression of fear (Coleman-Mesches and McGaugh, 1995a). In this experiment animals had either bilateral cannulas or unilateral cannulae implanted into the amygdala. The 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 2 days later. Some of the animals were retrained at this time and tested again 2 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 into the right or left amygdala did not affect acquisition. However, rats given a lidocaine infusion into the right amygdala were impaired on the retention of the task two days later (Coleman-Mesches & McGaugh, 1995a).

This study provides evidence for dynamic changes in the function of the rodent amygdala in the storage of fear based behavior. The data suggest that both the right and left amygdala functioning together are required for the acquisition of aversive memories. However, retention of these memories shifts to the right amygdala over time, once these memories are acquired (Coleman-Mesches & McGaugh, 1995b).

Hemispheric bias in changes in fear have also been found in models of anxiety associated with epilepsy. Adamec and Morgan (1994) have shown that kindling of the left medial/basolateral amygdala decreases anxiety (anxiolytic effect) while kindling the right hemisphere in analogous nuclei increase anxiety (anxiogenic effect). Moreover, it has been demonstrated that the anterior part of both the basolateral and central amygdaloid nuclei are important for conflict performance based on lesion studies and the infusion of benzodiazepines (Davis et al, 1994). A similar trend has been noted in our laboratory. Degree of anxiety following kindling is correlated with placement of the electrode in the anterior-posterior plane. Specifically, more anterior locations were associated with increased anxiety while more posterior locations were associated with a lower degree of anxiety (Adamec and Morgan, 1993; Adamec and McKay, 1993).

Our laboratory has found analogous phenomena in the cat. In this instance increased defensiveness towards rodents produced pharmacologically,

is accompanied by a long lasting potentiation of activity in the left and right amygdala-periacqueductal gray circuits. However, the potentiation in the left hemisphere decayed after 40 days, but the potentiation in the right hemisphere persisted as long as the behavior change. Together the data of this study suggest right hemisphere LTP of amygdala efferents is critical for increased fearfulness (Adamec, 1996).

Conclusions and Justification for the Present Study

Considering the above research a series of experiments has been designed to investigate several hypotheses. The first experiment will examine the effects of the benzodiazepine antagonist, Flumazenil (RO-15-1788) in an ecologically valid animal model of PTSD that was developed in our laboratory. The animals will be exposed to a cat for 5 minutes and their anxiety-like behavior (ALB) and startle behavior will be assessed one week later in the elevated plus maze and startle apparatus. Ten minutes prior to ALB and startle testing rats will be given Flumazenil or vehicle to test the effects of Flumazenil on the predator stress induced increases in ALB and startle.

This study will examine parallels between human studies of the effects of Flumazenil on affect in PTSD patients, and effects of Flumazenil in an animal model of this disorder. We have hypothesized that Flumazenil will have a similar effect in our animal model as it does in patients who suffer from

PTSD. Thus, we expect Flumazenil to have no effect on the anxiety levels of these animals.

The second set of experiments will study the role of NMDA receptors in the pathophysiology of anxiety. Increases in anxiety-like behavior in the rat following predator stress have been blocked by systemic injections of the non-competitive NMDA antagonist, MK-801 (0.30 mg/kg), given just prior to predator exposure (Adamec, Shallow, & Budgell 1996, Submitted to Journal of Psychopharmacology). The present experiment will extend this work by injecting MK-801 directly into the left or right basolateral amygdala or bilaterally into both hemispheres. Given the previous literature, we hypothesize that infusions of MK-801 into either the right hemisphere or bilaterally will prevent lasting increases in anxiety-like behavior in the elevated plus maze.

These studies, if successful, should provide clarification of the role of the NMDA receptor complex in the physiology of anxiety pathophysiology in general, and in PTSD in particular. From a clinical perspective it would be important to know by what mechanism, and where in the brain, changes take place which lead to increased anxiety and reactivity (startle).

METHODS EXPERIMENT 1

Experiment 1 was designed to investigate the properties of the benzodiazepine antagonist Flumazenil and the effects of exposure to a cat on anxiety and response to acoustic startle.

Subjects

A total of 80 Long-Evans rats (locally supplied by animal care services) weighing approximately 140-175 grams at the beginning of the experiment were used. Rats were individually housed in plastic transparent cages on metal racks which held a total of 20 cages. Animals were maintained on a 12 hour light-dark cycle with lights on at 07:00. Food and water were available *ad libitum* at all times.

Drugs

Flumazenil (RO15-1788) was suspended in the vehicle Tween-80. The suspension was prepared by mechanical mixing for a period of 15 minutes followed by a 15 minute period of ultrasonic dispersion with a sonicator. The injections of drug as well as vehicle were intraperitoneal (i.p) in a volume of 0.5 ml.

Handling

Animals were adapted to the laboratory for one week before the start of the experiment. Over this time they were handled three times. Handling consisted of picking up the animal with the gloved hand and gently restraining it on the forearm. Pressure was gently increased if the animal tried to escape. When the rat became still the grip was loosened. The rat was held in this position for one minute.

Groups

Animals were divided randomly into 4 groups of 20 animals each. Two of the groups received an i.p injection of Flumazenil (10 mg/kg) while the other two groups received an injection of the vehicle (Tween 80) before anxiety testing. One of the groups injected with Flumazenil as well as one of the vehicle groups was exposed to a cat for 5 minutes one week prior to anxiety testing. The two remaining groups served as controls and were handled, but not exposed to a cat on the day their yoked partner was exposed to a cat.

Cat Exposures

The cat exposure room is a large carpeted room equipped with speakers and 2 video cameras (see Adamec et al, 1980 for a full description of the testing room). Video equipment was located outside the room to allow for

recording of both the rat as well as the cat behavior. The cat was placed in the room first. Rats were then introduced to the room via a wooden box. The rat was then gently pushed into the testing room. At the time of entry the 5 minute test began.

Cats generally sniffed and investigated the rats and in some instances gently pawed the rats, but under no circumstances were the rats harmed. At the end of the 5 minute test the rats were placed back in their home cages and returned to their holding room where they remained unhandled for a 1 week period.

Behavioral Testing

Exactly 1 week after the cat exposure, all of the animals were tested for their level of "anxiety". Two of the groups, one cat exposed group and one control, received an i.p injection of Flumazenil (10 mg/kg) 10 minutes before behavioral testing. Two of the other groups, one exposed group and one control group, received an i.p injection of the vehicle, Tween 80, in a volume equal to the Flumazenil injections. The behavioral testing involved 5 minutes exposure to a hole-board followed by 5 minutes in the elevated plus maze. All behavior was videotaped for later analysis. After the rats were placed in the center of the holeboard, test timing began.

The hole board is a 60 by 60 cm square box which contains 4 evenly

spaced holes which the animals may explore. This apparatus provides an independent measure of activity and exploratory behavior. The measures taken from videotape were the number of times the rat investigated the holes (head dips) as well as the number of times the animal reared up (rears), and the time spent in any kind of activity (Time Active). After 300 seconds in the hole board the animals were gently placed in the center of the plus maze facing an open arm. The plus maze has two open arms and two closed arms in the shape of a plus sign. The arms are raised 50 cm above the ground. All of the arms in the maze are 10 cm wide by 50 cm long. In addition, the closed arms have walls of the same length which project upwards but do not close at the top. Both open arms of the maze have a 3cm high railing which followed along the edges of the arm.

Measures of anxiety in the elevated plus maze were ratio time and ratio entry. Ratio time is the amount of time the animal spends in the open arms divided by the total time spent in any of the arms. The smaller this ratio, the more "anxious" the animal is said to be. Ratio entry is the number of entries into the open arms divided by the number of entries into any of the arms. In a similar fashion the smaller this ratio the more "anxious" the animal is said to be. A measure of activity/exploration taken was total arm entries.

Two other measures taken in the elevated plus maze were Frequency and Time spent in Risk Assessment. Risk Assessment occurs when the animal

poked his head and forepaws into one of the open arms of the maze. The hindquarters of the animal remain in the closed arm of the maze. These values were divided by the amount of time spent in the closed arm of the maze to yield two new values, Relative Time and Relative Frequency Risk Assessment. These latter ratio measures were considered to be independent of time spent in the closed arms.

Acoustic startle

One day after the holeboard and plus maze testing, animals were tested for acoustic startle response. The apparatus (San Diego Instruments) was fitted with a 8" plexiglass cylinder which was used to hold the animal, as well as a speaker for producing the sound bursts. Motion of the animal within the cylinder was detected via a piezoelectric transducer which was positioned below the cylinder.

Animals were first acclimated to the apparatus under background conditions with noise set at the 80 decibel level for a period of 10 minutes. Directly following the acclimation period a test session was initiated which consisted of 80 noise bursts set at 110 decibels for a 20 msec duration with a 10 second inter-pulse interval. A computer attached to the apparatus recorded 80 of the samples for a 250 msec duration. Peak startle amplitude and time to peak within each of the trials was determined by the computer and saved

for later analysis. The startle parameters were set to evaluate the rate of habituation to acoustic startle stimuli for these animals. At the end of the startle session the animals were returned to their home cages.

METHODS EXPERIMENT 2

Subjects

Two hundred Long-Evans rats (locally supplied by animal care services) weighing between 200 and 250 grams at the start of the experiment were used. Rats were housed individually in transparent plastic cages on metal racks holding 20 cages. Animals were maintained on a 12 hour light-dark cycle with lights on at 07:00 hrs. Food and water were available ad lib at all times.

Handling

Animals were handled as in Experiment 1.

Groups

Animals were divided into a total of 10 groups with 20 animals in each group. Three of the groups had cannulas aimed at the right basolateral nucleus of the amygdala, three had cannulas aimed at the left basolateral amygdala, while three more of the groups had cannulas implanted bilaterally

into the basolateral amygdala. One of the groups served as an unoperated control. One group of the right amygdaloid placements received an injection of the non-competitive NMDA receptor antagonist MK-801 (10μg/0.5μl/side/min), (RBM), 30 minutes before exposure to a cat. Another group with right placements received an equivolume injection of sterile saline (RBS), 30 minutes before exposure to a cat. The remaining right placement group served as an operated control and received no injections (RBH) nor were these animals exposed to a cat. Left placement groups were treated similarly and were designated as LBM, LBS, and LBH. The bilateral groups were also treated similarly resulting in three separate groups denoted as BBM, BBS, and BBH. The bilateral animals received two separate injections of either MK-801 or saline, as appropriate. This was accomplished by infusing into the right hemisphere first followed directly by an infusion into the left hemisphere. All injections were completed 30 minutes before exposure to a cat. The handled groups were neither operated nor injected and were designated as HB.

Surgical Procedures

Animals were anaesthetized with sodium pentobarbital (Somnotol, 65 mg/kg) and implanted under aseptic technique with chronic guide cannulae

(Plastics One) aimed at the basolateral nucleus of the amygdala using the coordinates of Paxinos and Watson, 1986 (AP -2.30 ; ML +/- 4.80 ; V -7.50). Cannulas were held in place with dental acrylic cement which was secured in place with 4 stainless steel screws mounted to the skull. The patency of the cannulae was maintained by the use of dummy cannulas (Plastics One) which was flush with the tip of the cannula. Rats were then given the wide spectrum antibiotic Chloromycetin (Chloramphenicol, 10 mg/kg, s.c) to combat any infection. Animals were allowed to recover from the surgery for a 1 week period.

Drug Infusion Procedures

Animals in drug infusion groups were given MK-801 or an equivalent volume of sterile physiological saline. The drugs were contained in a 1.0 μl Hamilton syringe which was attached to an infusion pump (Sage Instruments). The syringe was in turn fitted with a connector (Plastics One) which was fitted with a 33 Gauge internal cannula (Plastics One) that protruded 1 mm below the tip of the guide cannula. The pump was adjusted to a setting which allowed the pump to deliver the required volume in 56 seconds. At the end of the 56 second time period the pump was shut off and the internal cannula left in place for 1 minute to allow for diffusion away from the tip. At the end of the infusion, the dummy cannula (Plastics One) was reinserted into the guide

cannula and the animal was returned to its home cage to wait the 30 minute period before exposure to the cat.

Cat Exposures

The rats were exposed to cats for 5 minutes in the room described in Experiment 1. The cat was put in the testing room first and the rat was put in the room via a wooden box with a sliding platform. After the rat entered the room, the 5 minute test was started. At the end of the exposure, animals were returned to their home cages where they remained unhandled until the start of the behavioral testing 1 week later. The animals that were designated as operated-handled (i.e RBH, LBH, BBH) and handled only (HB) were handled and returned to their home cages for 1 week before the behavioral testing.

Behavioral Testing

One week after the cat exposures animals were tested in the hole-board as well as the elevated plus maze, as described in Experiment 1. All behavior in both of the mazes was videotaped for later analysis.

Acoustic Startle

In this part of the experiment animals were subjected to two consecutive

days of startle. The first day of startle used the same parameters as the Flumazenil study (See Experiment 1). At the end of the startle session the rats were returned to their home cages until the following day. The second day of startle consisted of a 10 minute acclimation period with the background noise set at 60 decibels. The acclimation period was then followed by 20 trials with a burst intensity of 120 decibels and an inter-trial interval of 1 minute. All other aspects of the startle session were the same as detailed above. At the end of the startle session the animals were returned to their home cages.

Histology

At the end of the behavioral testing the animals were sacrificed. Animals were deeply anaesthetized with sodium pentobarbital (Somnotol, 70 mg/kg) and perfused transcardially with 0.9% saline and 10% formaldehyde. Brains were then sunk overnight in 30% sucrose. The following day coronal sections are cut on a cryostat at a thickness of 37 µm. Sections were subsequently stained with cresyl violet to allow for visualization of the cannula tips. Sections were then analyzed with an image analysis program (Jandel Scientific) to localize the tips of the cannulae. Coordinates (Paxinos and Watson) of the tips were measured with the image analyzer after a correction was made for tissue shrinkage.

Results

Results Experiment 1

Plus Maze/Holeboard Analysis. Data were analyzed by an Analysis of Variance (ANOVA). Independent variables were Drug (Flumazenil or Tween-80) and cat exposure. There were no Drug or Drug by Cat Exposure interactions for any measure in the elevated plus maze. However, there was a Cat Exposure effect on anxiety levels of the animals.

Rats exposed to a cat had lower ratio times than those not exposed to the cat ($F(1,75)=4.47$, $p< 0.038$). This effect was observed regardless of whether the animals were treated with Flumazenil or vehicle. Conversely, ratio entry demonstrated only a marginally significant difference in that control animals had higher ratios than animals that were cat exposed, $F(1,75) = 3.87$, $p < 0.053$ (Figure 1).

In addition, animals exposed to a cat showed less Risk Assessment than animals that were not exposed. Both the relative time spent engaging in Risk behavior as well as the relative frequency of such behavior were lower in cat-exposed rats than in non-exposed animals ($F(1,75) = 4.10$, $p < 0.047$; and $F(1,75) = 7.20$, $p < 0.009$, respectively, Figure 1). In addition, cat exposed animals showed fewer total entries into the arms of the maze than controls ($F(1,75) =4.82$, $p < 0.03$, Figure 1).

Before the plus maze data can be properly interpreted it must be determined if behavior in the plus maze is attributable to level of anxiety or exploratory tendencies. This can be accomplished by examining the hole board data.

First, the number of head dips was greater for animals given Flumazenil than vehicle injected controls ($F(1,75) = 5.79$, $p < 0.019$, Figure 1). In addition, there were more head dips exhibited by animals that were exposed to a cat than by animals which were not exposed ($F(1,75) = 4.84$, $p < 0.03$, Figure 1). It thus appears from the hole board data that exploratory behavior was increased by both cat exposure as well as Flumazenil injection prior to behavioral testing (Figure 1). There were, however, no significant effects of either drug injection or cat exposure on either the number of rears in the hole board, or the time active (Figure 2).

Analysis of Startle Data

The Jandel Table Curve Program was used to find a best fitting function for the change in startle amplitude over trials. Figure 2 shows the best fit exponential curve for all four groups combined. The plotted values are average startle amplitude values over 16 blocks of 5 trials/block. Individual startle amplitude values were first obtained by removing baseline startle amplitude

(Vstart) from the peak startle amplitude (Vmax) of each block (Vmax-Vstart). The Vstart and Vmax values were determined by the computer for each trial following the acoustic stimulus within the 250 msec sampling period.

Flumazenil had no effect on the rate of habituation of the groups (Duncan Test). In contrast animals that were exposed to cats habituated more slowly. This was determined as follows. Exponential curves were fitted to the average startle amplitude of individual animals in each of the 4 groups. All fits were good (df adjusted r^2 =0.917 to 0.962). From these fits, an average trial constant (τ) was determined for each group. This constant represented the number of trials required for startle amplitude to decay to 67% of maximum. Trial constants for both cat- exposed groups (Flumazenil-Exposed, FE ; Tween-80-Exposed, TE) were greater than the non-exposed groups (Flumazenil-Not Exposed, FN ; Tween-80-Not Exposed, TN, Duncan test, $p<.05$, variances of the τ values were used to construct the error term for the Duncan test).

The effects of cat-exposure and Flumazenil on startle amplitude (Vmax-Vstart) per se were also examined. Data were averaged over blocks 1 through 7 and over blocks greater than 10 (see Figure 2). The first block range was chosen because block 7 was 2 standard deviations above the mean τ value for the cat exposed animals. Block 9 was initially excluded from the analysis as a rebound in amplitude occurred at that point. A later analysis showed that

this block was not significantly different from data recorded in blocks 10 through 16.

Analysis of variance was used to compare all groups with respect to mean startle amplitude averaged over blocks 1-7, 8-9 and 10-16. There was a marginally significant group effect for the block 1-7 data ($F(3,556) = 2.60$, $p<0.052$, Figure 2). The non-exposed vehicle group (TN) was contrasted to the other three groups under the Bonferroni criterion and found to be less than the mean scores of the other 3 groups, which did not differ ($t(556) = 2.43855$, $p < 0.015$). From the data it appears that cat exposure increased the startle amplitude over the first seven blocks in the FE and TE groups. In addition, animals given Flumazenil but not exposed to a cat also had elevated startle amplitudes equal to exposed groups. In contrast, groups did not differ in amplitude collapsed over blocks 10 through 16 ($F(3, 556) = 0.89$, $p < 0.45$), or blocks 8 and 9 ($F(3, 1276) = 1.85$, $p < 0.14$.

Results Experiment 2

Comparison of Operated Controls and Handled Only Controls

Analysis of variance compared the 3 operated groups with the

unoperated handled controls to determine if the surgery had an effect on the behavior of the animals. None of the groups differed on any of the measures in the holeboard or the plus maze. Therefore, cannulation of the amygdala, per se, had no effect on the animals' behavior.

Body Weight

Due to the fact that animals were treated in a number of different ways it was important to determine whether or not body weight was affected. At the time of surgery all of the animals were statistically equivalent in weight (308g ± 3.00 mean weight ± SEM for all groups). Weights were then expressed as a percentage of the mean surgery weight one week later at time of Cat Exposure and two weeks later at the time of Behavior Testing (Mean ± SEM at cat exposure = 108.8% ± 1.5% ; Mean ± SEM at behavioral testing = 114% ± 0.8%). Groups did not differ at either of these times.

Effects of Injection and Cannula Placement

A two-way analysis of variance using both drug and cannula placement factors was performed to compare the effects of the drug on the behavior of the animals in the holeboard and plus maze. There were three levels of Drug

(operated handled and not exposed, vehicle prior to cat exposure, MK-801 prior to cat exposure). Placement also had three levels (left, right, bilateral). A main Drug effect only was found for Ratio Time, ($F(2,171) = 4.79$, $p < 0.01$), with no significant interaction. Multiple comparison mean contrasts (Duncan test, $p < 0.05$) showed that the operated handled controls had a significantly higher score than both the vehicle and the MK-801 groups which did not differ from each other (see Figure 3). Hence, cat exposure increased anxiety equally in animals given MK-801 and vehicle.

Relative frequency of risk behavior was not normally distributed (Omnibus $k^2 = 170.09$, $p < 0.001$). Therefore, the non-parametric Kruskal-Wallis one way ANOVA on rank sums was done testing the drug effects across all the cannula placement groups. A significant overall difference was found, $\chi^2(2) = 7.65$, $P < 0.03$, Figure 4). Animals that were injected with the inert vehicle Tween-80 and exposed to a cat showed less risk assessment than the animals that were operated and handled only. The animals that were given MK-801 fell between the operated handled animals and those animals treated with the vehicle (Kruskal-Wallis Multiple Comparison test, $p < 0.05$, Figure 4).

Since MK-801 partially blocked the effects of cat exposure, it was of interest to know if this effect varied with hemisphere of placement. Separate Kruskal-Wallis multiple comparison were done contrasting handled, vehicle, or MK-801 groupings with either right, left or bilateral placements.

Interestingly, animals that were injected with either vehicle or MK-801, in the right hemisphere, did not differ significantly, but scored significantly lower than their operated controls, ($p< 0.05$, Figure 4). Moreover, rats given vehicle injections in the left hemisphere or bilaterally and exposed to a cat had significantly lower scores than their unexposed controls, ($p< 0.05$, Figure 4). More importantly, however, was the observation that animals given MK-801 in the left hemisphere or bilaterally did not significantly differ from either the vehicle group or the operated handled controls.

Analysis of Startle Data

80 Trial Startle: Day 1

Data were analyzed as in Experiment 1. Each group of animals was tested for any changes over trials individually to simplify the analysis. The first 9 groups which included Right, Left, and Bilateral (MK-801+Exposed, Saline+Exposed, Operated+Handled) did not change over the 16 Blocks of 5 trials/block, Figure 5. In contrast, the Handled-Only group (Group 10) did change over blocks of trials ($F(15,319) = 1.85$, $p < .03$, see Figure 5). The decline over blocks of startle amplitude fit a declining exponential function with a Trial constant of 4.32 (DF adj $r^2 = 0.679$, see Figure 6).

Since the first 9 groups did not differ over the 16 blocks, data were

collapsed across all 80 trials for each animal of each group. Data were not normally distributed (Omnibus $k^2 = 68.63$, $p < 0.001$), so the non-parametric Kruskal Wallis ANOVA was used to compare groups 1-9 ($\chi^2(8) = 12.33$, $p<.138$, df=8). Though the 9 groups did not differ in the overall Kruskall Wallis analysis, multiple median comparisons were done on groups within each hemisphere in view of the Flumazenil study results. Animals given either MK-801 or saline in the right hemisphere and exposed to a cat displayed higher median startle amplitudes than animals implanted in the right amygdala, but not exposed (Figure 7). Conversely, animals that were implanted in the left amygdala or bilaterally and handled, or cat exposed and given vehicle or MK-801, did not differ. Therefore cat exposure increased startle only in right hemisphere rats. Implants in the left hemisphere seem to interfere with the effects of cat exposure on startle amplitude.

In addition to the median startle amplitude analysis, the time required to reach maximum amplitude was also analyzed for the operated groups in each hemisphere and bilaterally. There were no changes in the time to maximum startle amplitude over trials or in time to maximum startle amplitude collapsed over trials (Groups 1-9), Figure 7.

A further analysis contrasted the first 9 groups with the unoperated handled animals (Group 10) on median startle amplitude. Group comparisons were done separately for trial blocks 1-16 because Group 10 (Unoperated)

showed habituation over blocks, whereas the remaining groups did not. Only on trial 1 did the groups differ. The unoperated handled animals had a higher startle amplitude, ($F(9, 190) = 2.00$, $p < .05$), than all other groups except the cat exposed bilateral group receiving MK-801, (Duncan Test, $p<.05$). This group (BBM) fell between Group 10 and the other 8 groups. There were no group differences on any other trial.

20 Trial Startle:Day 2

Twenty four hours after the 80 trial startle paradigm, animals were exposed to a 20 trial startle test. There were no trial effects for any of the groups. For group contrasts Kruskal-Wallis One Way Anova on Ranks was used because the data were not normally distributed (Omnibus test, 119.41, $p<.001$). Individual median contrasts were done with the Kruskal-Wallis multiple z test. Figure 8 shows startle data collapsed over trials for the first 9 groups. In the right hemisphere, Handled-Implanted and MK-801+Exposed animals did not differ, but showed lower startle amplitudes than right amygdala rats given saline and exposed ($p < .05$). In the left hemisphere, animals given MK-801+ exposed showed significantly lower startle amplitudes than saline + exposed animals or the operated control rats which did not differ from each other. There were no differences between the groups

implanted bilaterally.

Operated groups were also compared with respect to the time required to reach the maximum startle amplitude. There were no trial effects or differences between any of the groups (Figure 8).

Anatomical Localization of Cannula Implants

An analysis was first done of the coordinates of cannula tip placements of bilateral animals in the three stereotaxic planes to determine if there were any differences between placements in the two hemispheres. There were no differences detected so coordinate data were averaged across each hemisphere for each plane. These averaged data were then used in the following analysis.

A two-way Analysis of Variance was done with both drug and placement as two factors. There were three levels of drug (Operated -Handled and Not-Exposed, Vehicle 30 minutes before Exposure, MK-801 30 minutes before Exposure). Placement also consisted of three levels (Left, Right, Bilateral). Placement data were the cannula placements in mm for each of the three spatial planes (Anterior-Posterior AP, Medial-Lateral ML, and Dorsal-Ventral DV). There were no significant differences noted in either the cannula placements or the drug groups. Furthermore there were no significant interactions. Hence, behavioral effects could not be attributed to the

placement of the cannula.

Positions of the injection cannulae were projected onto plates from the rat atlas of Paxinos and Watson (1986) (Figures 9-12). Plates used ranged from -2.30 mm to -3.14 mm posterior to bregma. A series of 4 plates within this range was used. Lateral and ventral coordinates of cannula placements whose AP planes ranged from 0.0 mm to -2.42 mm posterior to bregma were averaged and plotted on the plate AP -2.30 mm posterior to bregma. In a similar fashion, lateral and ventral coordinates of cannula placements extending from -2.43 mm as far as -2.67 mm posterior to Bregma were averaged and plotted on the -2.56 mm plate. Coordinates of placements which were further back in the range of -2.68 mm to -2.96 mm from Bregma were also averaged and plotted on the -2.82 mm plate. Any placements which were beyond -2.96 mm posterior to Bregma were averaged and plotted on the plate which was -3.14 mm posterior to bregma. To display the position of the cannula tips, an ellipse with hatch marks was utilized to illustrate the 95% confidence intervals of both the average Medial-Lateral and Dorsal-Ventral planes. From the plates it can be seen that most of the tips fell within the lateral to mediolateral nuclei of the amygdala.

Discussion

The Effect of Flumazenil and Cat Exposure on Rat Anxiety

In agreement with our original hypothesis there were no Flumazenil effects on anxiety-like behavior in the elevated plus maze. However, the data did demonstrate that exposure to a cat increases anxiety in rodents at one week after the exposure. This is in agreement with earlier data from our laboratory which demonstrated a long-lasting anxiety in a rat exposed to a cat for 5 minutes (Adamec and Shallow, 1993). There was, however, no interaction between cat exposure and the administration of either Flumazenil or vehicle. It was shown that animals exposed to a cat yielded lower ratio time and ratio entry values than animals that were not exposed. The lower these index scores, the more "anxious" the animals are said to be.

Two other measurements associated with the plus maze, Relative Time Risk and Relative Frequency Risk also were affected by cat exposure, consistent with previous work (Adamec & Shallow, 1993). Animals that were exposed to a cat displayed less frequency and less time engaging in Risk-behavior. Again, there were, no drug effects on these measures nor was there

any interaction between cat exposure and drug. In line with a reduction of Risk-behavior, cat-exposed animals also demonstrated fewer total entries into the arms of the maze than their non-exposed controls. There was no effect of Flumazenil on these measures nor was there any interaction between exposure and the drug.

For these results to be considered specific to anxiety, the effects of cat exposure on the exploratory tendencies of the animals must be shown to be independent of effects on measures of anxiety. Analysis of the hole board data demonstrated this to be the case. Head dipping in the holeboard is considered to be a measure of exploratory behavior (File & Wardill, 1975). If exposed rats explored the open arms less due to reduced exploratory motivation, then the number of head dips should be reduced in these exposed animals. This was not the case. In the present study the number of head dips in the hole board was greater for cat-exposed animals than for animals that were not exposed. Moreover, Flumazenil also increased head dipping.

The Effect of Flumazenil and Cat Exposure on Startle

Several interesting results emerged from the startle analysis. First, cat-exposure increased the magnitude of response to auditory startle stimuli. This pattern of results parallels the startle responses of PTSD patients. Human studies have demonstrated an exaggerated response to startle stimuli

(Kolb 1987; Butler et al, 1990).

Analysis of the rate of habituation demonstrated that groups of rats exposed to a cat habituated more slowly than those groups not exposed to cats. Animals that were exposed to a cat had higher trial constants than animals that were not exposed. Since the trial constant reflects the number of trials required to reach a 67% decay of startle amplitude it can be said that the exposed animals took longer to habituate to the stimuli. There is a parallel to these findings in human PTSD sufferers. Orr et al (1995) examined habituation of acoustic startle induced skin conductance change in PTSD patients and in controls. Magnitude of the response decreased quickly in the control subjects but was significantly slower in the PTSD patients. In addition controls and PTSD patients showed similar levels of startle at the end of the testing session. This pattern was also seen in the present study, an equivalent startle amplitude endpoint was reached by all of the groups by the end of block 8 of the 16 blocks of 5 trials.

In contrast, Flumazenil had no effect on the rate of habituation in these animals, nor was there any interaction between Flumazenil and cat-exposure. The fact that Flumazenil had no effect on anxiety has parallels in the clinical literature as well. Nutt (1995) found that Flumazenil was unable to evoke any PTSD symptoms in PTSD patients nor did it alter their anxiety levels. Similarly, Randall and colleagues (1995) found that there were no differences

in response to Flumazenil or placebo in PTSD patients.

Although Flumazenil had no effect on anxiety as measured in the plus maze, it did affect the magnitude of the startle response in animals not exposed to a cat. In these animals, Flumazenil produced higher startle amplitudes than the vehicle controls. This result suggests Flumazenil was exerting an anxiogenic effect with respect to startilability, but only in rats not exposed to a cat. However, Flumazenil was behaviorally neutral in exposed animals. This pattern of findings is consistent with a model proposed by File and Hitchcott (1990). In their model, the action of Flumazenil is dependent upon the behavioral state of the animal. This behavioral state is seen as moving on a continuum oscillating between an anxious state and a non-anxious state. Accordingly, when the animal is anxious, Flumazenil acts as an anxiolytic, conversely, if the animal is not anxious, Flumazenil exerts an anxiogenic effect. In effect Flumazenil acts to drive the existing state toward some state midway between the extremes.

Using this model, it is assumed that all non-exposed animals were at the non-anxious end of the continuum. Hence, the vehicle non-exposed group (TN) which was neither exposed nor injected with Flumazenil should have been at the lowest startle amplitude level, which is what was observed. In contrast, the Flumazenil non-exposed group (FN), showed a greater startle response because Flumazenil was anxiogenic, driving their state toward the more

anxious end of the anxiety continuum. This movement toward a more anxious state appeared as an increase in the startle amplitude.

The model also applies to the behavior of the exposed animals. Both the FE and the TE groups were exposed to the cat. Their level of anxiety was increased by an equivalent amount along the anxiety continuum. The administration of Flumazenil to the FE group did not increase startle beyond that produced by cat exposure. This suggests that cat exposure drove the system to a point midway between the extremes. At this level of function, Flumazenil would not have any behavioral effects.

Taken together, the data from the Flumazenil study support the hypothesis. Traumatic predator stress induced increases in plus maze anxiety is not affected by Flumazenil. Negative results, must of course, be interpreted with caution. It is possible that a wider dose range of Flumazenil might produce effects on plus maze anxiety in cat exposed rats. Nevertheless the dose of Flumazenil used in the study was not too low to produce behavioral effects. Flumazenil did increase the startle response in rats not exposed to cats. On the other hand, the drug had no effect on startle already amplified by cat exposure. This finding is a more positive result which also suggests Flumazenil has no effect on anxiety as measured by startle acoustic amplitude in cat exposed rats.

The pattern of findings parallel those reported in PTSD patients. As

such this study provides further validation of the cat exposure paradigm as a model of anxiety produced by traumatic stress in PTSD patients.

One use of an animal model is to explore causal mechanisms. Toward this end, the second part of this thesis examined the role of NMDA receptors in the amygdala in the increases in rat anxiety produced by predator stress.

NMDA Antagonists and Anxiety

This part of the project was designed to evaluate the role of NMDA receptors in the pathophysiology of anxiety with application to the generalized anxiety associated with PTSD. We hypothesized that local infusion of an NMDA antagonist, such as MK-801, into the right amygdala or bilaterally into the basolateral amygdala would be able to prevent the long-lasting anxiety produced by cat-exposure.

As reported previously, cat-exposure produced a long-lasting increase in anxiety in rodents as measured in the elevated plus maze (Adamec & Shallow, 1993). However, the injection of MK-801 into the lateral amygdala did not prevent the lasting increase in anxiety as assessed by Relative Time spent in the open arms of the maze (Ratio Time).

There are several possible explanations of why MK-801 was ineffective in blocking the increase in anxiety as measured by Ratio Time. One is that

the cannulas were in the wrong amygadaloid nuclei. Most of the cannulas were in the lateral and mediolateral nuclei as opposed to the basolateral nucleus. The majority of the literature concerning aversive memory cite the basolateral nucleus as the critical nucleus (Campeau et al, 1992 ; Coleman-Mesches and McGaugh, 1995a,b). Another possibility is that the dose used was unable to occupy enough receptors to have an effect. It is plausible that, of the different indices of fearfulness, Ratio Time is the least sensitive to NMDA receptor manipulation. It is probable that blockade of a full complement of receptors is required to effect a change in this index. A recent study from our laboratory supports this view. Decreases in Risk Assessment were blocked with either 0.16 mg/kg or 0.30 mg/kg MK-801 i.p, however, 0.30 mg/kg was required to block the decreases in Ratio Time (Adamec, Shallow & Budgell, 1996, Submitted to Journal of Psychopharmacology).

Consistent with this view is the observation that MK-801 partially blocked the effects of cat exposure on Relative Frequency of Risk behavior. Animals that were given MK-801 in the left hemisphere or bilaterally had Relative Frequency Risk scores which fell midway between the operated controls and vehicle treated animals. Furthermore, these data indicate that this effect is mediated by the left hemisphere, suggesting a lateralization of function. The idea of laterality of amygdala function is supported in the literature (Coleman-Mesches & McGaugh, 1995a,b; Adamec & Morgan, 1994).

The implication of the left hemisphere in control of change in Risk Assessment is a novel result, in that previous work on different indices of rat defensive behavior point to the importance of the right amygdala. Together these data suggest that there are separate neural substrates in different hemispheres mediating the different indices of fearfulness in rodents.

There are several explanations for a partial block of anxiety by MK-801 of Risk Assessment. Cannula placement may be important. Since most of the cannulas were implanted in the lateral nuclei as opposed to the basolateral nucleus it is possible that the placements were not optimal. Further, it may be possible that the dose of the drug was insufficient to block the required number of receptors, especially if the cannulas were some distance from the critical receptors.

NMDA Antagonists, Cat Exposure and the Startle Response

Exaggerated startle response is a consistent symptom of patients with PTSD. This study replicated an earlier finding, that a 5 minute exposure of a rat to a cat is sufficient to increase the magnitude of the acoustic startle response one week after the exposure. Animals in this part of the study were exposed to two separate sessions of startle testing. The first day of testing involved the presentation of 80 evenly spaced acoustic bursts (every 10 secs) at 110 dB. The startle parameters allowed habituation to occur to permit

determination of the rate of habituation. The second day of testing involved the presentation of 20 evenly spaced acoustic bursts (every minute) at a higher intensity (120 dB) to produce a response which did not habituate.

The 80 trial startle session yielded some interesting results. First, the parameters used did produce habituation as expected, but only in unoperated, handled controls. None of the operated groups showed any habituation. Therefore, cannula placement in the amygdala of either hemisphere interfered with habituation to startle. One reason for this was a reduction in startle amplitude produced by cannula placement. Unoperated rats showed greater startle amplitudes than implanted rats on the first block of 5 trials. Thereafter they did not differ from operated rats.

Despite these effects of damage, cat exposure did increase startle amplitude to these startle parameters, but only in rats with cannulas in the right hemisphere. Cannulation of left or left+right amygdalas prevented the effect of cat exposure. The left lateral amygdala, therefore, is implicated in increased startle response following cat exposure. Neither damage to the right lateral amygdala by cannulation, nor injection of MK-801 had any effect on the increase in startle amplitude produced by cat exposure. Therefore, the right lateral amygdala does not appear to participate in predator stress induced increases in startle at these parameters.

The importance of the left amygdala in predator stress induced increases

of startle amplitude was seen in the 20 trial higher intensity stimulus paradigm, as well. Cannulation of left or left+right amygdalas prevented the increase in startle following cat exposure. Interestingly, MK-801 in the left amygdala prior to cat exposure reduced startle amplitude measured one week later relative to handled or vehicle+exposure groups. It is unclear what this means, though it does suggest intensity of the acoustic stimulus and/or rate of presentation may differently engage NMDA receptor-mediated processes.

A similar dependence of NMDA processes on intensity and rate of acoustic stimulus was seen in the right amygdala. In the 20 trial experiment, as in the 80 trial experiment, rats with cannulas in the right amygdala and injected with vehicle at cat exposure showed increased startle amplitudes one week later. In contrast to the 80 trial study, MK-801 in the right amygdala in the 20 trial study blocked the effects of cat exposure (see Figures 7&8). These findings suggest a very selective engagement of NMDA receptor dependent processes in the right lateral amygdala which is dependent on higher intensity and/or slower rate of presentation of acoustic stimuli. The higher decibel level (120dB) was probably required to activate NMDA systems within amygdaloid circuitry that participates in lasting change of response to startle stimuli.

The decrease in startle amplitude produced by MK-801 in the left lateral amygdala also implicates NMDA receptors in increases in startle produced by

cat exposure. However, since damage to the left amygdala interfered with the effects of cat exposure, it cannot be concluded with confidence how NMDA processes participate in the left amygdala in stress induced increases in startle amplitude.

These conclusions apply to the amplitude of the startle response. There were no differences between any of the groups with respect to time to reach maximum startle amplitude in either the 80 trial session or the 20 trial session at the higher decibel level. These data suggest that cat exposure is affecting the amplitude of the startle response but not the speed required to reach it. It further suggests that the NMDA receptors of the amygdala are not involved in regulating the speed of the response.

Implications For Post Traumatic Stress Disorder

The results further validate the cat- exposed rat as a model of PTSD. It has been shown that cat-exposure increases anxiety as measured by the elevated plus maze. Further, it has been shown that Flumazenil has no effect on the anxiety levels of these animals in the plus maze, a result that has clinical parallels. Nutt(1995) has shown that Flumazenil does not exacerbate or create any PTSD symptomatology in PTSD patients. The present study also demonstrated an exaggerated startle response in animals that were exposed

to a cat. Analogous findings are also documented in the clinical literature. Butler et al (1990) has shown that patients with PTSD show an exaggerated eyeblink response to startle stimuli. In the present study, exposed animals habituate to acoustic startle more slowly than controls. In addition, all of the animals, whether they were exposed or not reached an equivalent startle amplitude end point by the end of the study. Both of these findings have parallels in the human literature. Orr et al (1995) found that the magnitude of the response declined quickly in control subjects, but was considerably slower in PTSD patients. Moreover, both groups of subjects reached the skin conductance non-response criterion by the end of the session.

Conclusions

Data from this study suggest that lasting change in anxiety and fearfulness in rodents is mediated by more than one neural substrate. The study further suggests that both hemispheres as well as NMDA receptors are differentially involved in the expression of this anxiety. In the case of startle, the involvement of the amygdala and NMDA receptors appears to be acoustic stimulus parameter dependent. Further work with this model is needed to

develop some of the intricacies that are apparent in this system. It is hoped that the development of this model will open up new avenues for the treatment of post traumatic stress disorder.

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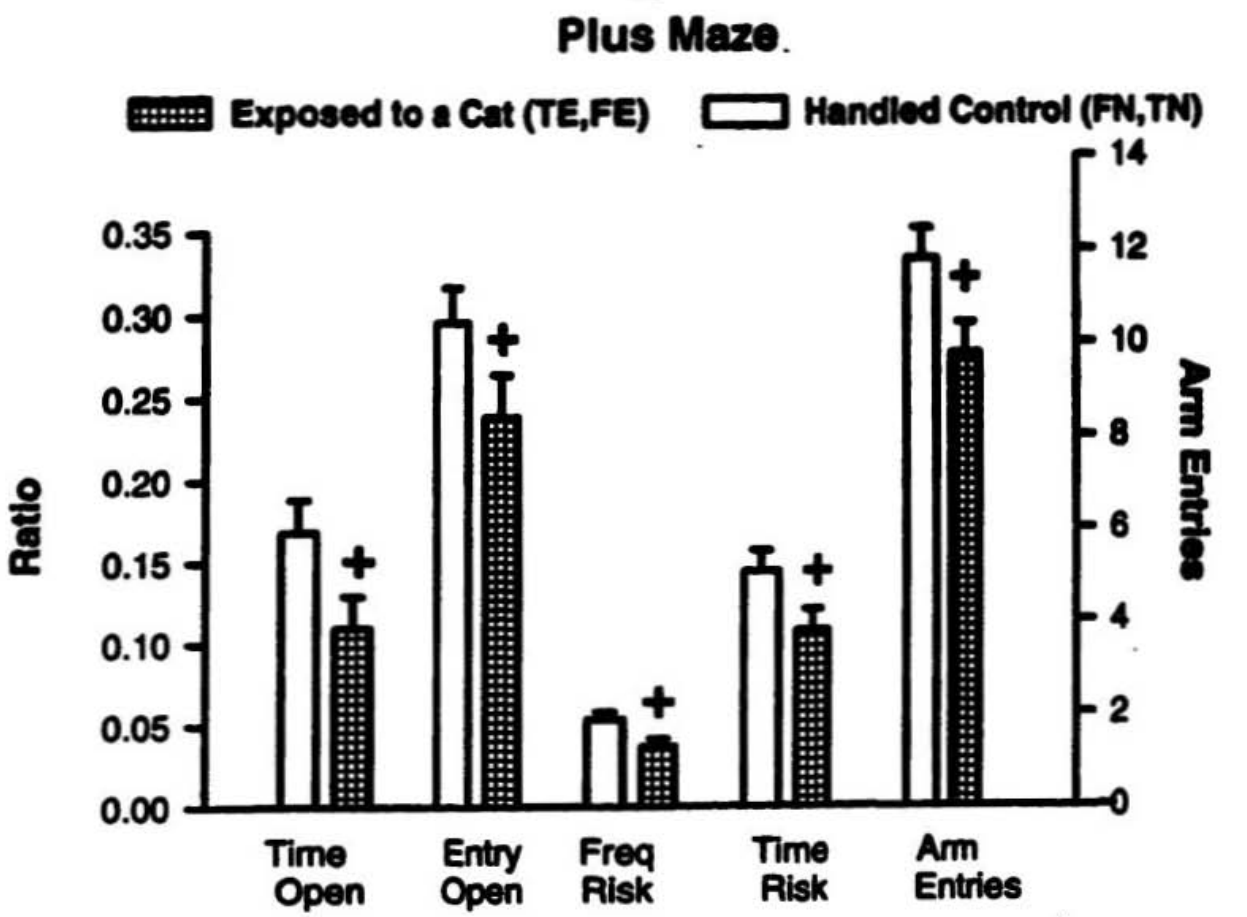
FIGURES


Plus Maze
Exposed to a Cat (TE,FE)
Handled Control (FN,TN)
Ratio
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Arm Entries
14
12
10
8
6
4
2
0
Time Open
Entry Open
Freq Risk
Time Risk
Arm Entries

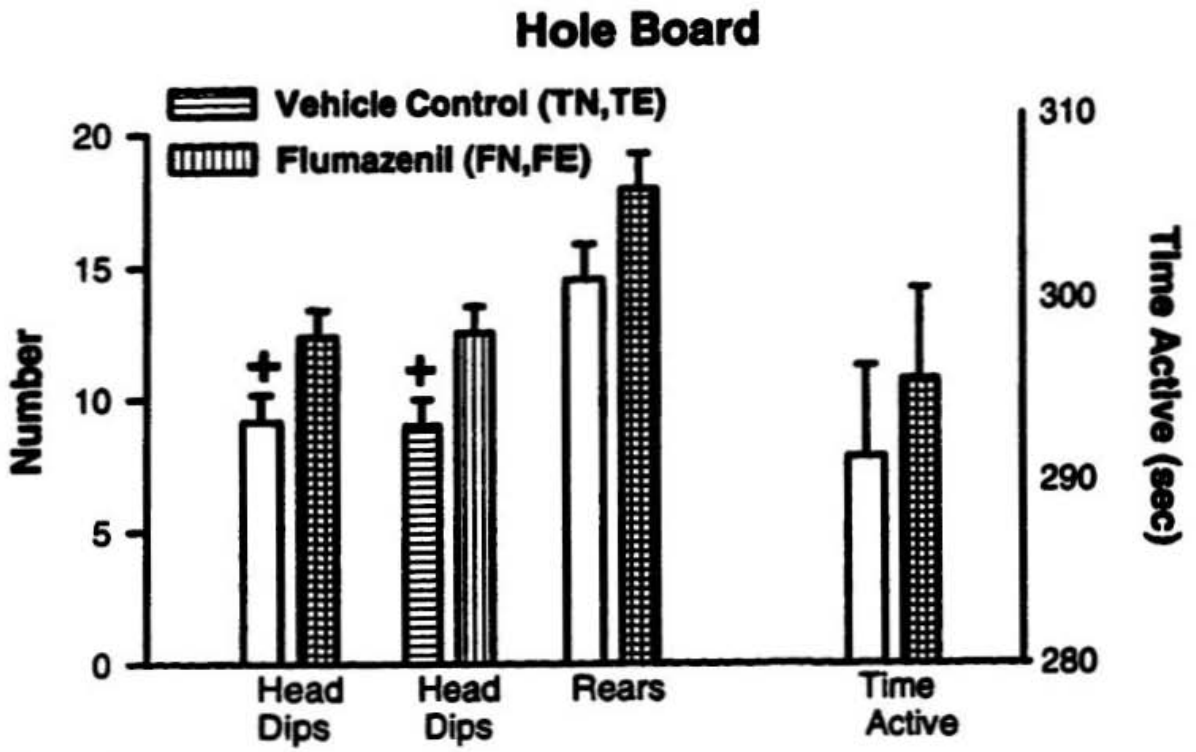
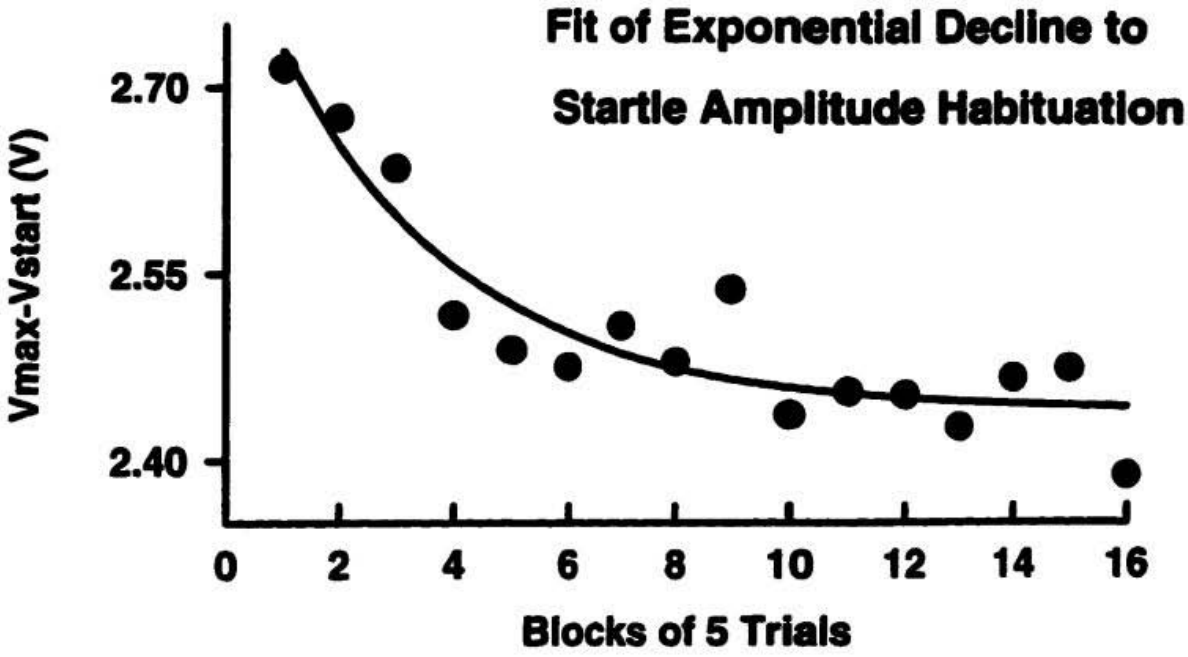

Hole Board
Vehicle Control (TN,TE)
Flumazenil (FN,FE)
Number
20
15
10
5
0
Time Active (sec)
310
300
290
280
Head Dips
Head Dips
Rears
Time Active

Figure 1


Fit of Exponential Decline to
Startle Amplitude Habituation
Vmax-Vstart (V)
2.70
2.55
2.40
0
2
4
6
8
10
12
14
16
Blocks of 5 Trials

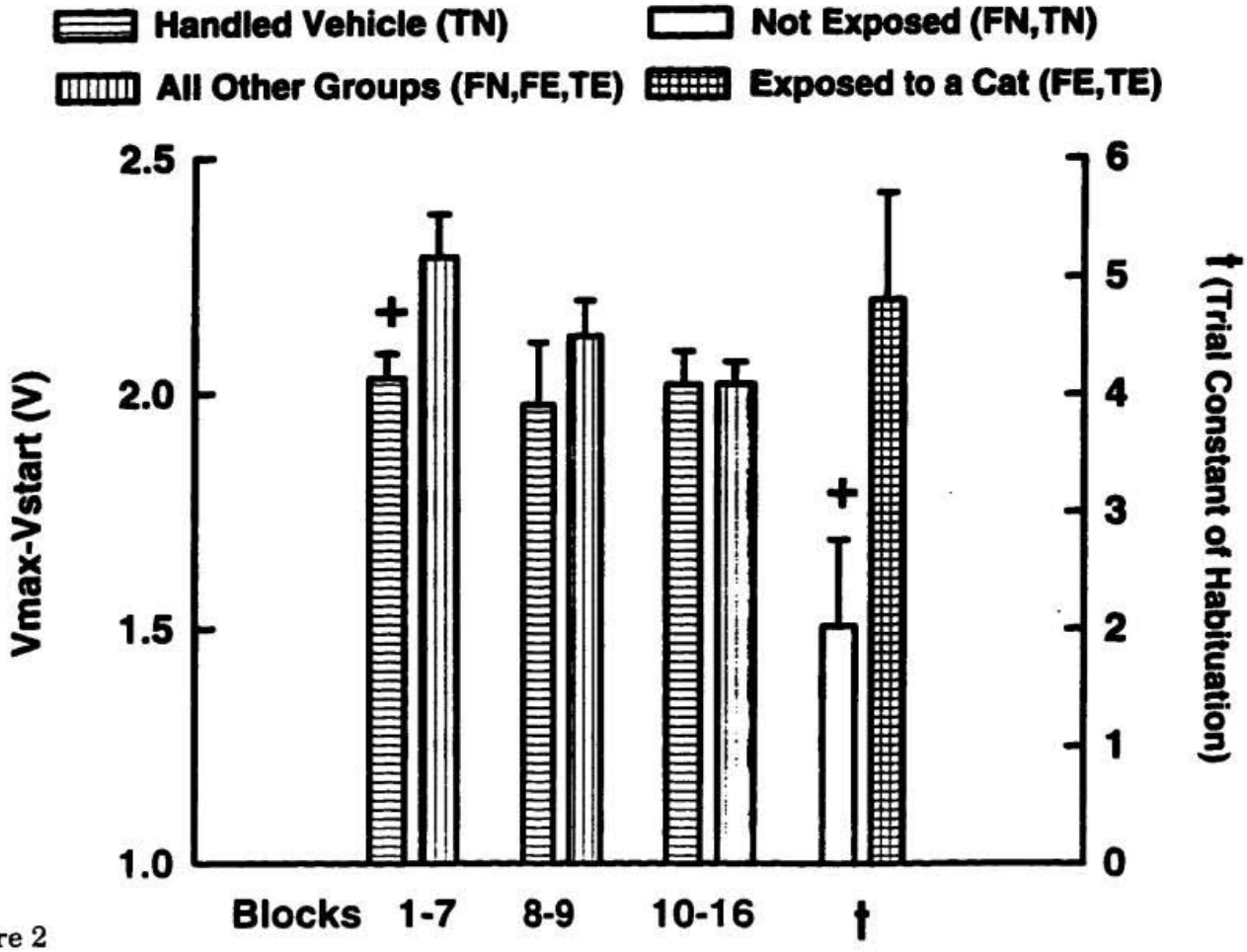

Handled Vehicle (TN)
Not Exposed (FN,TN)
All Other Groups (FN,FE,TE)
Exposed to a Cat (FE,TE)
Vmax-Vstart (V)
2.5
2.0
1.5
1.0
6
5
4
3
2
1
0
† (Trial Constant of Habituation)
Blocks 1-7
8-9
10-16
†

Figure 2

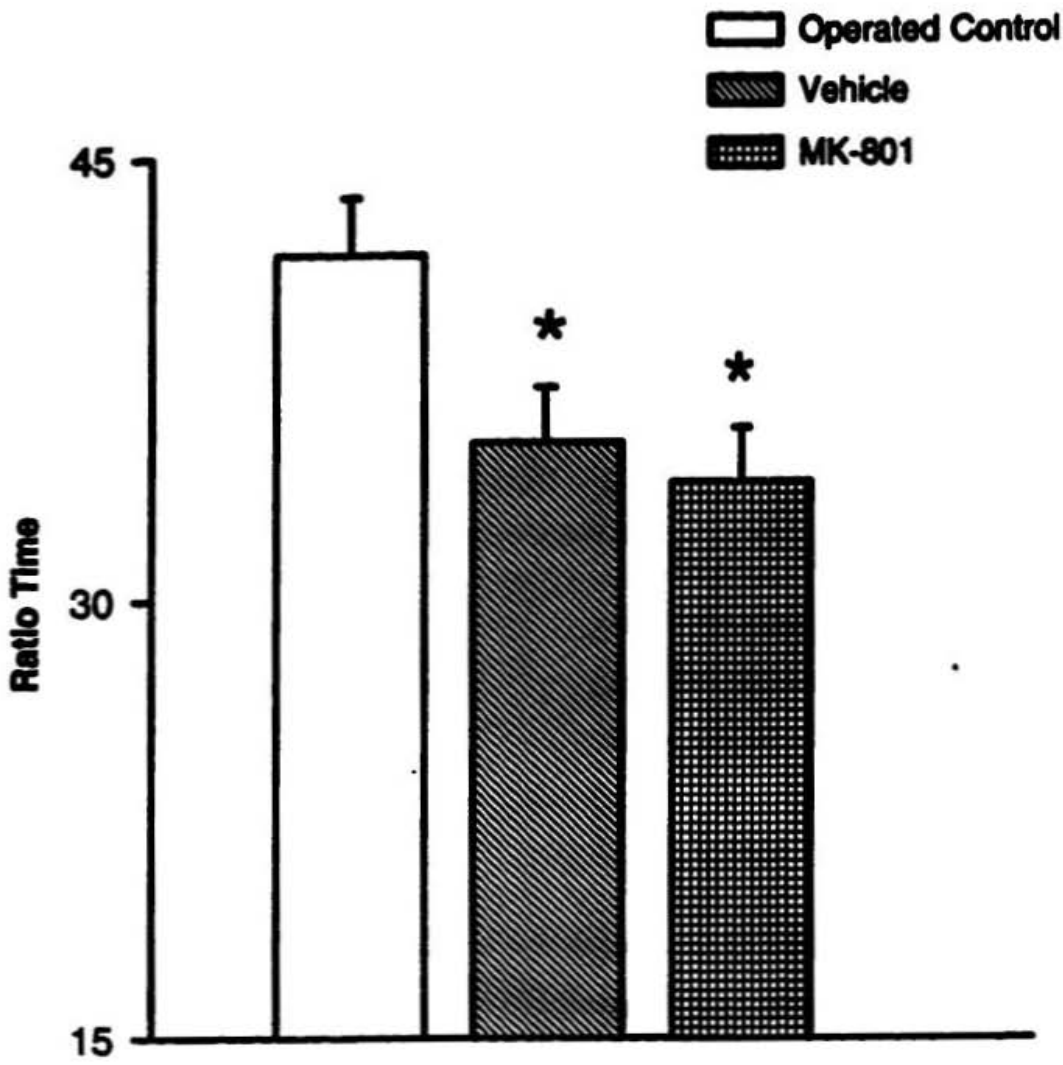


Figure 3

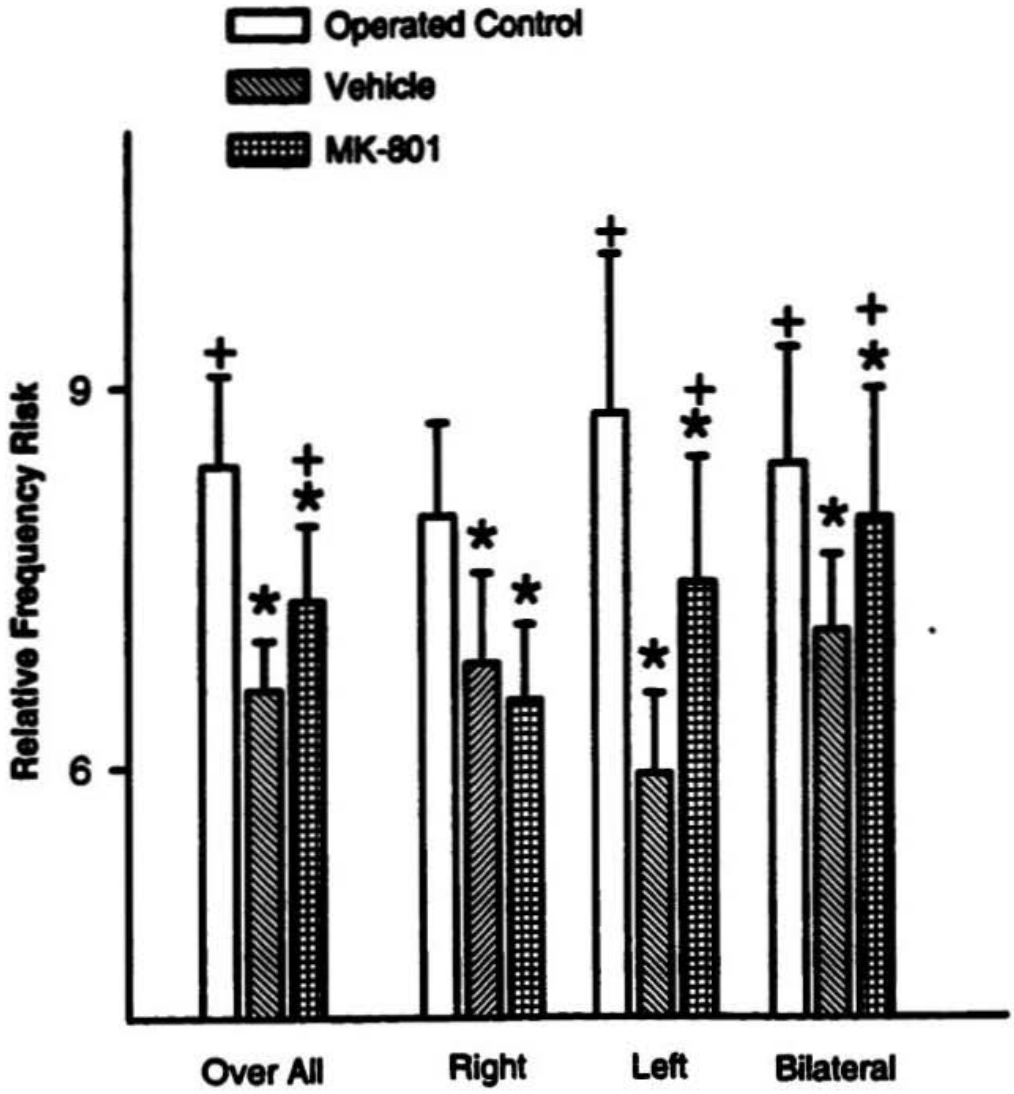

Operated Control
Vehicle
MK-801
Relative Frequency Risk
9
6
Over All
Right
Left
Bilateral

Figure 4

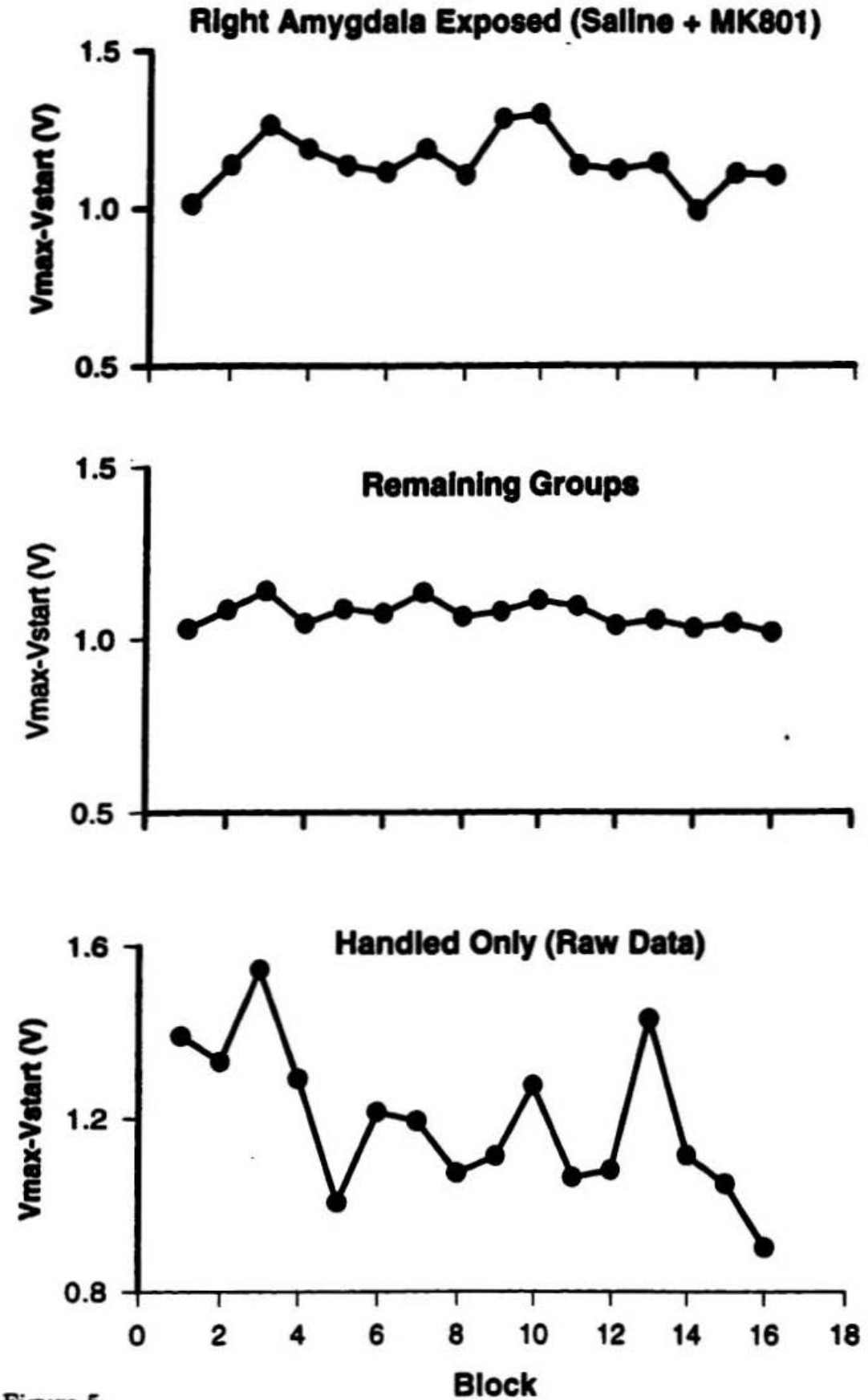

Right Amygdala Exposed (Saline + MK801)
Vmax-Vstart (V)
1.5
1.0
0.5
Remaining Groups
Vmax-Vstart (V)
1.5
1.0
0.5
Handled Only (Raw Data)
Vmax-Vstart (V)
1.6
1.2
0.8
0
2
4
6
8
10
12
14
16
18
Block

Figure 5

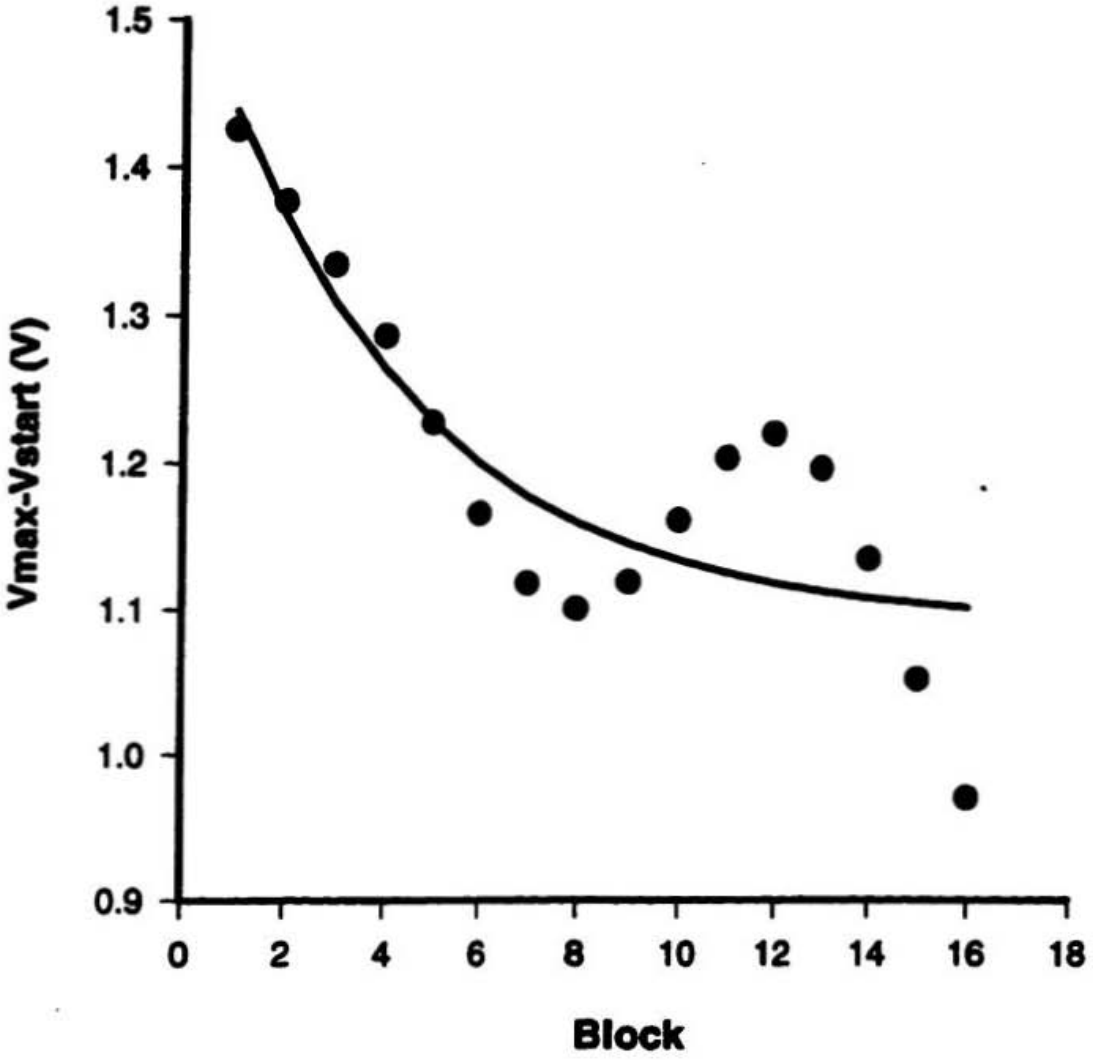


Figure 6

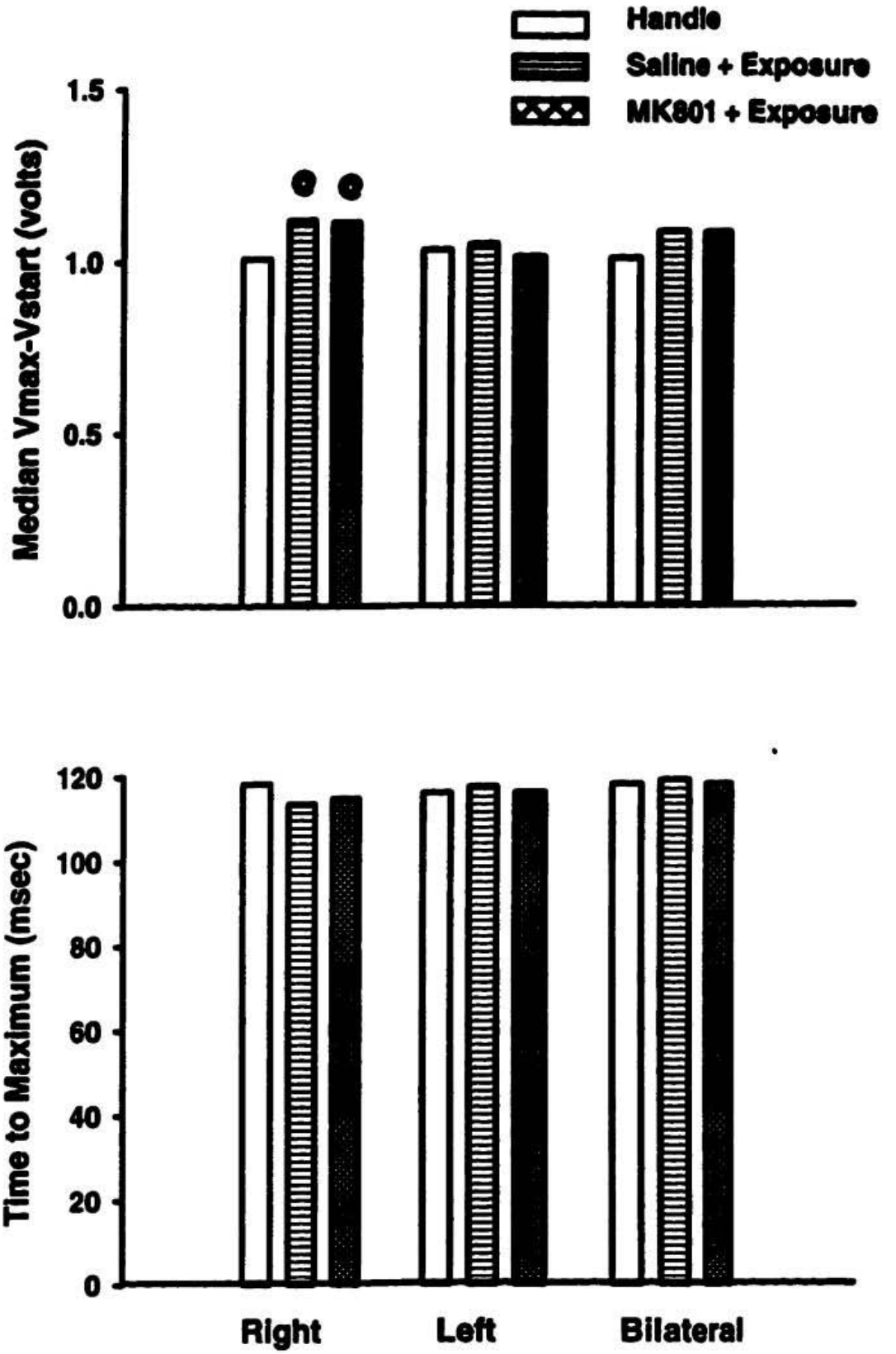

Handle
Saline + Exposure
MK801 + Exposure
Median Vmax-Vstart (volts)
1.5
1.0
0.5
0.0
Time to Maximum (msec)
120
100
80
60
40
20
0
Right
Left
Bilateral

Figure 7

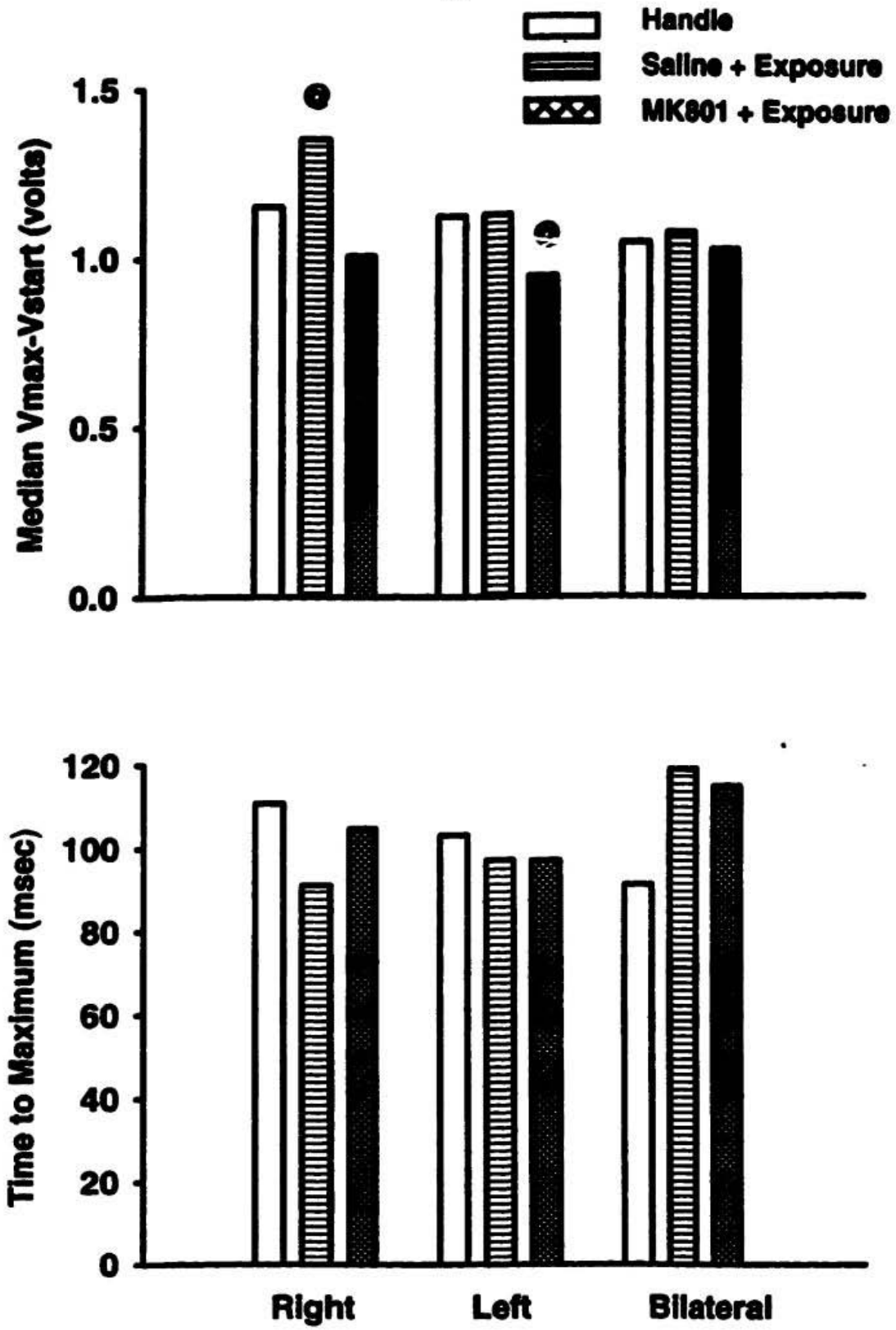

Handle
Saline + Exposure
MK801 + Exposure
Median Vmax-Vstart (volts)
1.5
1.0
0.5
0.0
Time to Maximum (msec)
120
100
80
60
40
20
0
Right
Left
Bilateral

Figure 8

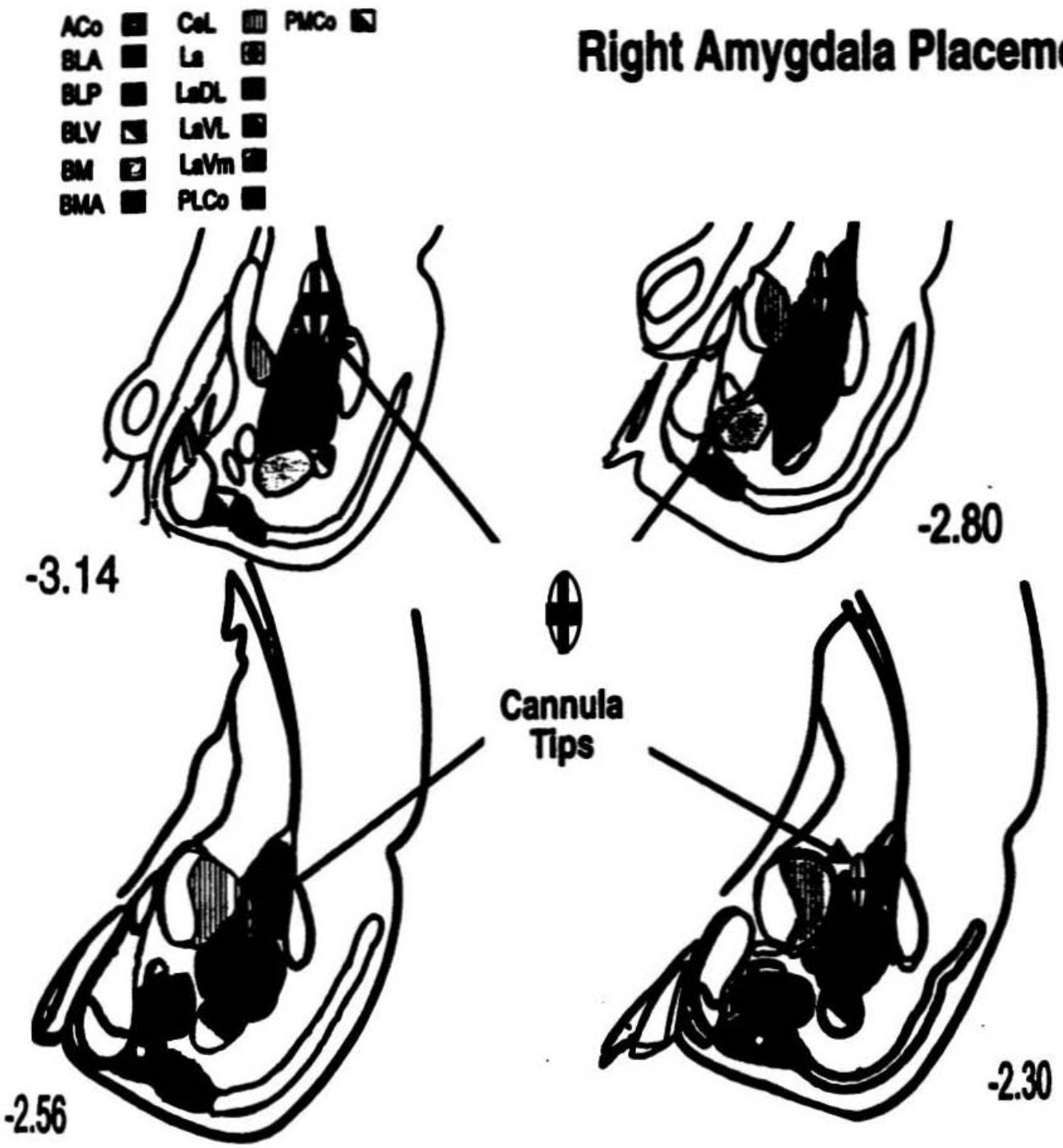

ACo
BLA
BLP
BLV
BM
BMA
CeL
La
LaDL
LaVL
LaVm
PLCo
PMCo
Right Amygdala Placements
-3.14
-2.80
Cannula Tips
-2.56
-2.30

Figure 9

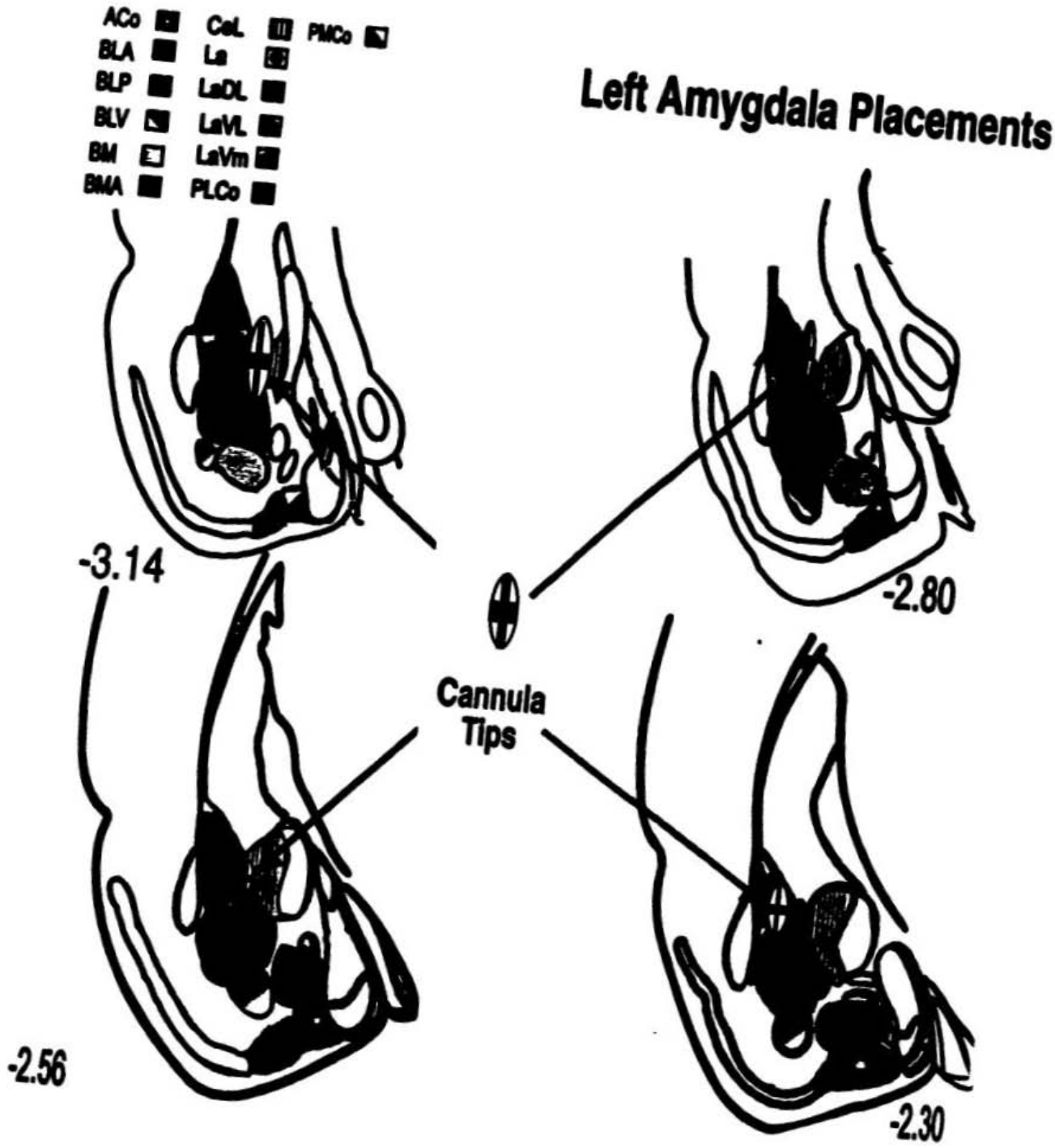

ACo
BLA
BLP
BLV
BM
BMA
CeL
La
LaDL
LaVL
LaVm
PLCo
PMCo
Left Amygdala Placements
-3.14
-2.80
Cannula
Tips
-2.56
-2.30

Figure 10

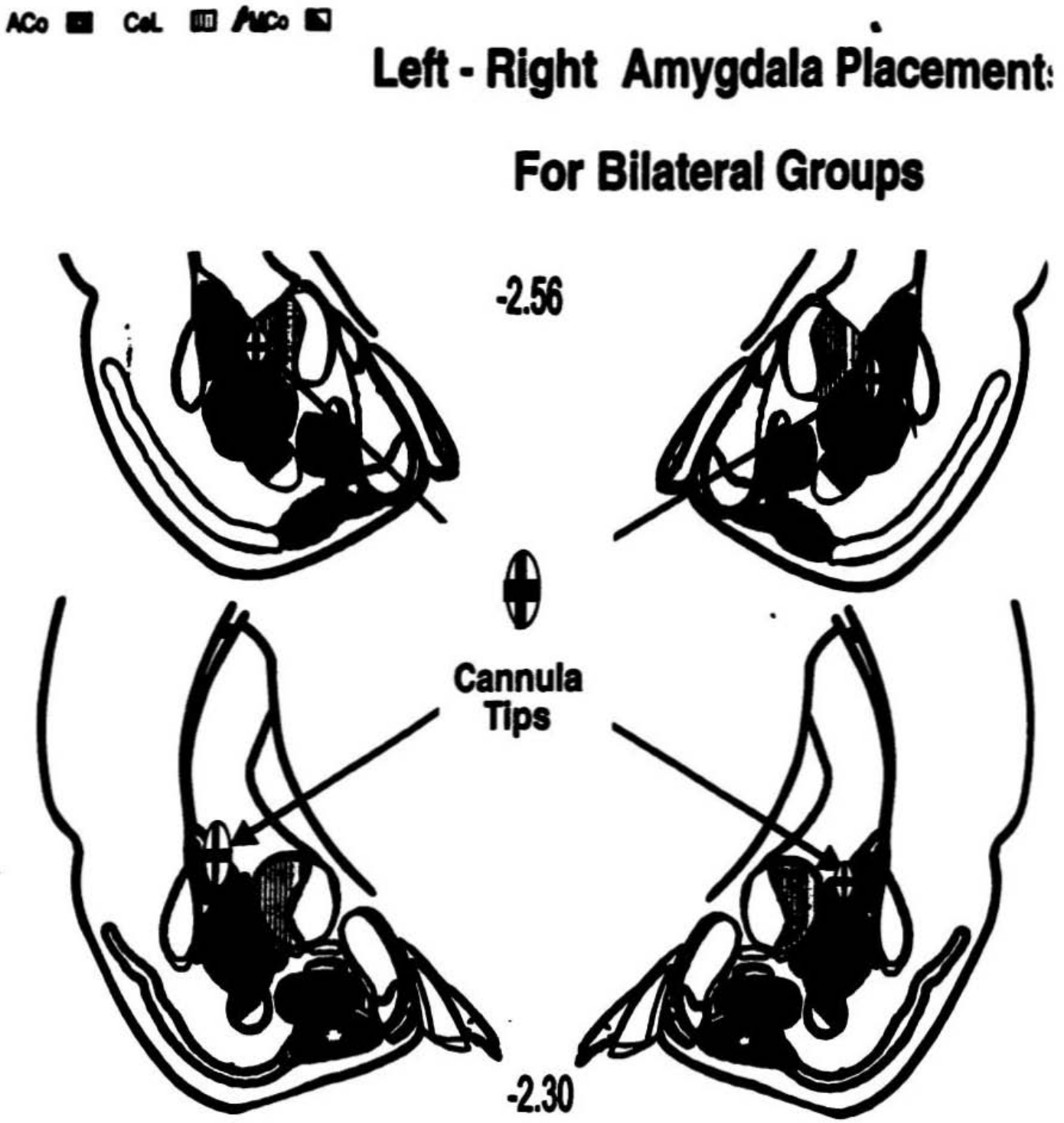

ACo
CeL
AHCo
Left - Right Amygdala Placement
For Bilateral Groups
-2.56
Cannula
Tips
-2.30

Figure 11

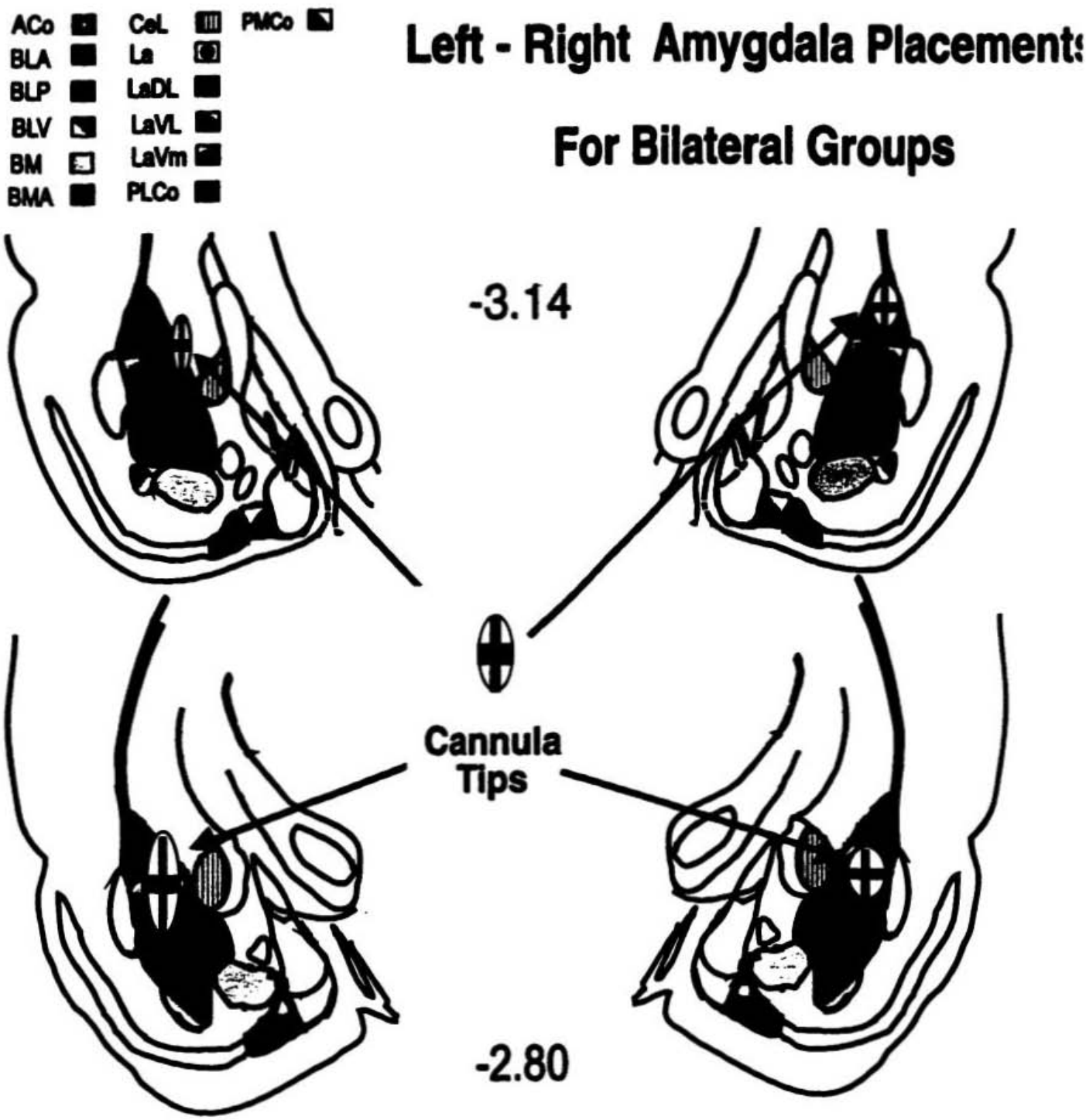

ACo
BLA
BLP
BLV
BM
BMA
CeL
La
LaDL
LaVL
LaVm
PLCo
PMCo
Left - Right Amygdala Placements
For Bilateral Groups
-3.14
Cannula Tips
-2.80

Figure 12