STRESS-ACTIVATED HORMONAL RESPONSE FOLLOWING PREDATOR STRESS MEDIATES THE PRECIPITATION OF LONG LASTING CHANGES IN AFFECTIVE BEHAVIOR IN RATS

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Stress-Activated Hormonal Response following Predator Stress Mediates the Precipitation of Long Lasting Changes in Affective Behavior in Rats

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Abstract

The role of β-adrenoreceptors, glucocorticoid receptors (GRs), and mineralocorticoid receptors (MRs) in the consolidation of changes in anxiety-like behavior following predator stress were studied by injecting specific receptor blockers 1 minute after the stress event and testing for behavior change in a battery of tests 1 week later. Propranolol dose dependently blocked stress effects in all behavior tests except startle. GR block (RU486) alone was ineffective, but in combination with low dose propranolol blocked stress-induced anxiety-like behavior in all tests. Surprisingly, MR block (spironolactone) also prevented the consolidation of anxiety-like behavior in all tests except the light-dark box.

Startle results were complicated by the presence of both stress-induced increases and decreases in peak startle amplitude. Treatment with chlordiazepoxide and RU486 effectively blocked startle suppression, but not enhancement, indicating that the consolidation of these two memory processes may be mediated by different mechanisms. In contrast, when administered in combination with propranolol, RU486 prevented stress-induced startle enhancement, as did MR block using spironolactone.

Predator stress delayed habituation to startle in all rats. This was blocked by post-stress treatment of spironolactone, chlordiazepoxide, and RU486 + propranolol in combination, but not by RU486 or propranolol given alone.

Present findings indicate that consolidation of predator stress-effects share neurochemical mechanisms in common with fear conditioning models, and are relevant to the study of stress-induced changes in affect in humans.

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1. Introduction

Severe stress is a surprisingly common experience, with 50-60% of the North American population encountering a traumatic event in their lifetime (Kessler, Sonnega, Bromet, Hughes & Nelson, 1995). Systematic study of such experiences is necessary, as affective disorder may follow severe stress in as many as 15% of those exposed (Kessler, McGonagle, Zhao, Nelson, Eshleman & Wittchen et al, 1994). In fact, in light of recent tragedies such as the terrorist attacks on New York City, the need to study stress-induced affective disorders (such as post-traumatic stress disorder) has never been greater.

1.1. Post-Traumatic Stress Disorder (PTSD)

PTSD is a psychological condition resulting from exposure to a traumatic event. It first appeared in the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM) in 1980, and has a lifetime prevalence of 7.8% among American adults (Kessler, Sonnega, Bromet, Hughes & Nelson, 1995). As indicated in the DSM-IV, it is possible to experience traumatic stress without manifesting PTSD. However, in cases where the emotional effects of severe stress do not subside, a diagnosis of PTSD is made. To receive such a diagnosis, a person must both:

(i) experience, witness, or be confronted with an event or events that involved actual and/or threatened death, serious injury, or a threat to the physical integrity of self or others

(ii) respond with intense fear, helplessness, or horror

The resulting symptoms of PTSD are diverse and varied, and are represented as three distinct clusters in the DSM-IV, each having its own characteristics and defining features. The three clusters are:

(i) *Intrusion*: the traumatic event is persistently re-experienced through (1) recurrent thoughts, images, or perceptions of the event (2) distressing dreams about the event (3) feelings of reliving the event (4) intense psychological distress to cues that symbolize the event, and (5) physiological reactivity to those same cues

(ii) *Avoidance*: stimuli associated with the traumatic event are consistently avoided, and a numbing in general responsiveness is observed. Avoidance is indicated by the presence of three or more of the following symptoms: (1) efforts to avoid thoughts, feelings, or conversations associated with the trauma (2) efforts to avoid activities, places, or people that promote recollections of the trauma (3) inability to recall important aspects of the trauma (4) diminished interest or participation in previously enjoyable activities (5) feelings of detachment or estrangement from others (6) restricted range of affect, and (7) sense of a foreshortened future

(iii) *Hyperarousal*: a state of nervousness characterized by two or more of the following symptoms: (1) difficulty falling or staying asleep (2) irritability or outbursts of anger (3) difficulty concentrating (4) hypervigilance, and (5) exaggerated startle response

For a diagnosis of PTSD, such disturbances must endure for at least one month, and cause clinically significant distress or impairment in either social, occupational, or other important areas of functioning. PTSD can be further classified as acute (symptoms lasting less than 3 months), chronic (symptoms lasting greater than 3 months), or delayed onset (symptom onset at least 6 months after the stressor) (American Psychiatric Association, *DSM-IV*, 1994).

1.2. Animal Models of PTSD

Aspects of PTSD can be modeled in animals, which is important when the ethical and technical constraints involved in working with human subjects are considered. One such model is classical fear conditioning in rodents, in which an innocuous conditioned stimulus evokes a fear response after having been paired with a noxious unconditioned stimulus. This paradigm has implicated a role of the amygdala in the consolidation of fear memories in the brain. Acquisition of conditioned fear requires NMDA-dependent processes localized within the amygdala (Campeau, Miserendino & Davis, 1992; Davis, 2002; Bauer, Schafe & Ledoux, 2002), and rats that have acquired a conditioned fear response show long-term potentiation of neural transmission in amygdala circuitry (Rogan, Staubli & Ledoux, 1997; Blair, Schafe, Bauer, Rodrigues & Ledoux, 2001; Schafe, Nader, Blair & Ledoux, 2001). Such findings have spawned clinical interest in fear conditioning as a model of PTSD onset, and this model has been used clinically to develop prophylactic intervention strategies following traumatic stress with some success (Pitman, Sanders, Zusman, Healy, Cheema, Lasko et al, 2002).

As such, classical fear conditioning is the conventional model used for the study of traumatic stress. However, it fails to model generalized anxiety, which is also a feature of PTSD (Pitman, 1997). Therefore, animal paradigms capable of modeling such changes are necessary for the study of stress-induced affective disorder (Pitman, 1997), and to extend and validate the findings of fear conditioning models.

Generalized anxiety manifested as sensitized fearfulness can be modeled in animals through unprotected exposure to a species-relevant life-threatening event. Such models also represent an ecologically valid method for inducing affective psychopathology following stress. For example, unprotected and inescapable exposure to a cat (predator stress) lastingly increases rodent anxiety, and represents one model of sensitized fearfulness (Adamec, 1997; Adamec & Shallow, 1993; Cohen, Zohar & Matar, 2003). Following predator stress, behavioral changes are detectable in several tests of rodent anxiety such as the elevated plus maze, light dark box, social interaction test, and acoustic startle chamber. Increases in anxiety-like behavior have been measured using the above tests up to three weeks following exposure to a predator (Adamec, 1997; Adamec & Shallow, 1993). Such long lasting anxiety is clinically important, as it parallels the lasting nature of PTSD in human patients. Other clinically relevant changes seen following predator stress include differential vulnerability to stress effects (Cohen & Zohar, 2004), increased startle amplitude (Adamec, 1997), and delayed habituation to startle (Adamec, 1997). Similar effects have also been reported following exposure to cat odor alone, although on a much shorter timescale. This could suggest that stressor

intensity is a factor in the consolidation of anxiety-like behavior following stress (Cohen, Zohar & Matar, 2003; Cohen, Zohar, Matar, Zeev, Loewenthal & Richter-Levin, 2004).

1.3. Adrenal Stress Hormones and Emotional Memory

Memory consolidation following stress is important with respect to PTSD, as persisting memories of the trauma are characteristically seen in PTSD patients. A large body of evidence has implicated adrenal stress hormones, namely epinephrine and glucocorticoids, in the long term memory consolidation of an emotionally arousing event. Thus, the actions of these hormone systems at various sites in the brain are likely involved in the consolidation of traumatic memories in PTSD. (Elzinga & Bremner, 2002).

1.3.1. Catecholamines and Noradrenergic Involvement

It is well documented that centrally-released norepinephrine has a role in learning and memory (Ferry, Roozendaal & McGaugh, 1999b). Interestingly, systemic administration of epinephrine given either directly before or directly following an avoidance task enhances retention for that task in a dose dependent fashion (Introini-Collision & McGaugh, 1986). Furthermore, injections of the β-adrenoreceptor agonist clenbuterol have resulted in similar effects (Introini-Collision, Saghafi, Novack & McGaugh, 1992). Epinephrine-induced memory enhancements are blocked by administration of the β-adrenoreceptor antagonist propranolol (Introini-Collision et al., 1992; Sternberg, Korol, Novack & McGaugh, 1986), as are the effects of clenbuterol (Introini-Collision et al., 1992), implicating the β-adrenoreceptor in the consolidation of memory. Conversely, clenbuterol-induced memory enhancement is not blocked by administration of peripheral

acting β-adrenoreceptor antagonists (Introini-Collision & Baratti, 1986), indicating that the effects of epinephrine on memory storage involve activation of central β-adrenoreceptors. This may seem unlikely, as epinephrine cannot pass the blood brain barrier. However, it has been shown to mediate effects on memory consolidation through stimulation of a peripheral-central neuronal pathway (McGaugh & Roozendaal, 2002). Epinephrine activates β-adrenoreceptors of the vagus nerve which then ascend to noradrenergic cell groups in the nucleus of the solitary tract (NTS) (Clark, Smith, Hassert, Browning, Naritoku & Jensen, 1998). Here, numerous forebrain structures are innervated, and in this way the memory enhancing effects of epinephrine in the brain are directed (van Bockstaele, Colago & Valentino, 1996).

The amygdala is one brain structure which receives noradrenergic activation by the NTS, and thus participates in the memory-enhancing effects of peripherally-administered epinephrine. These effects are blocked by intra-amygdala infusions of the β-adrenoreceptor antagonist propranolol (Liang, Chen & Huang, 1995), whereas posttraining infusions of norepinephrine or clenbuterol (β-adrenoreceptor agonist) result in dose-dependent improvements in retention (Roozendaal, Koolhaas & Bohus, 1993). Taken together, it appears that activation of β-adrenoreceptors in the amygdala is important for the consolidation of memory following an emotionally arousing event.

More specifically, infusions of norepinephrine into the BLA improved memory for a spatial learning task, whereas infusions of propranolol, a β-adrenoreceptor antagonist, impaired memory for the same learning exercise (Hatfield & McGaugh, 1999). A number of other studies have confirmed that norepinephrine modulates memory through selective

activation of β-adrenoreceptors in the BLA (McGaugh, Introini-Collision, Cahill, Castellano, Damaz & Parent, 1993; Ferry, Roozendaal & McGaugh, 1999a), although α-adrenoreceptors are also involved (Ferry, Roozendaal & McGaugh, 1999a).

1.3.2. Corticosteroids and HPA Involvement

Ordinarily, glucocorticoid (cortisol in humans, corticosterone in rats) secretion follows a pronounced circadian rhythm, with peak levels at the onset of the active phase of the diurnal cycle (Keller-Wood & Dallman, 1984). Glucocorticoid concentrations are also elevated following perceived stress, as the brain attempts to coordinate an appropriate behavioral and metabolic response (de Kloet, Joels & Holsboer, 2005). At the core of the endocrine stress system is the Hypothalamic-Pituitary-Adrenocortical (HPA) axis, which controls blood concentrations of corticosteroid hormones through a negative feedback loop. Neurons of the hypothalamic paraventricular nucleus synthesize corticotropin releasing hormone (CRH) which stimulates the release of adrenocorticotropin releasing hormone (ACTH) from the anterior pituitary. ACTH is then released into systemic circulation, and initiates glucocorticoid synthesis upon binding the adrenal cortex (Whitnall, 1993). The adrenocortical stress response is terminated via a feedback signal mediated by glucocorticoid receptor (GR) binding in the limbic system (Sapolsky, Krey & McEwen, 1984). Additionally, glucocorticoid levels can undergo "delayed" feedback by way of gene transcriptional changes following chronic and prolonged stress (Herman, Ostrander, Mueller & Figueiredo, 2005; Pearce & Yamamoto, 1993).

Glucocorticoids also bind with mineralocorticoid receptors (MRs), which act alongside the GRs as primary regulators of the HPA axis. Although both substrates bind glucocorticoids, they differ with respect to distribution in the brain, and affinity for ligand (Trapp, Rupprecht, Castren & Reul, 1994). MRs are found primarily in limbic neurons, with the greatest density in hippocampus (Herman, Figueiredo, Mueller, Ulrich-Lai, Ostrander, Choi et al, 2003). These receptors have an affinity for corticosterone 10-fold higher than do GRs, and remain 80% occupied, even during glucocorticoid troughs of the circadian cycle (Reul & de Kloet, 1985). As such, MRs regulate the basal activity of the HPA system, and enhance adrenocortical secretion following stress (de Kloet, 1991; Ratka, Sutanto, Bloemers & de Kloet, 1989). In contrast, low affinity GRs are bound only during circadian peaks or in response to stress when glucocorticoid concentrations are high. In this way, GR occupancy acts as a termination signal to stress reactions through activation of the HPA feedback loop (de Kloet, Vreugdenhil, Oitzl & Joels, 1998). Together, the GR and MR form part of a dynamic system capable of controlling the stress response by monitoring and controlling changes in the level of circulating corticosterone. Such changes are also capable of affecting memory consolidation processes for an emotionally arousing event (McGaugh & Roozendaal, 2002; Roozendaal, 2000).

Unlike studies examining the role of norepinephrine in memory consolidation, the effects of glucocorticoids have not been consistent (Roozendaal, 2002). In some cases, glucocorticoid-induced memory enhancement has been reported (Buchanan & Lovallo, 2001; Sandi, Loscertales & Guanza, 1997), whereas others have shown interference with memory consolidation processes (Lupien & McEwen, 1997). This discrepancy can be

explained when the memory phase tested, and glucocorticoid dosage are taken into account (Roozendaal, 2000). Glucocorticoid-induced memory effects follow an inverted-U shape dose response curve, with moderate doses of corticosterone producing the greatest enhancement in memory for an inhibitory avoidance task when administered immediately after training (Roozendaal, Williams & McGaugh, 1999). The same effects have been observed using the synthetic glucocorticoid dexamethasone, and in a variety of other avoidance learning exercises (Roozendaal, 2000). Conversely, following adrenalectomy, and the consequent removal of endogenous corticosterone, rats show profound memory impairment when tested in a spatial learning task (Oitzl & de Kloet, 1992). However, learning impairments produced by adrenalectomy can be reversed through post-training injections of dexamethasone, further implicating glucocorticoid activation in memory consolidation of an inhibitory avoidance learning task (Roozendaal, Portillo-Marquez & McGaugh, 1996).

The presence of GRs in the amygdala has been demonstrated (Morimoto, Morita, Ozawa, Yokoyama & Kawata, 1996), and glucocorticoids are believed to exert an effect on memory consolidation through activation of the amygdala. As with norepinephrine, subregions of the amygdala appear to have preferential involvement in this process. Lesioning the BLA eliminates the memory enhancing effects of dexamethasone given systemically post-training (Roozendaal, Nguyen, Power & McGaugh, 1999). However, lesions to the ACE have no effect on dexamethasone-induced memory enhancement (Roozendaal, Nguyen, Power & McGaugh, 1999), suggesting that an intact BLA is required for glucocorticoid-mediated memory enhancement. Further support for this view

is provided from infusion studies. Post-training infusions of RU28362, a GR agonist, enhance retention for an inhibitory avoidance task when administered into the BLA, but not when infused into ACE (Roozendaal & McGaugh, 1997). The GR antagonist RU38486 produced opposite effects; impairing spatial memory when infused into the BLA (Roozendaal & McGaugh, 1997). Taken together, these findings suggest that glucocorticoid-induced memory enhancement is mediated through the binding of GRs in the BLA.

1.3.3. Glucocorticoid – Norepinephrine Interactions

With both glucocorticoid- and norepinephrine-induced memory enhancement localized in the BLA, studies were designed to investigate the relationship between the two systems. It has since been unequivocally shown that glucocorticoid-induced memory enhancement in the BLA depends on the integrity of the β-adrenergic system (Quirarte, Roozendaal & McGaugh, 1997; Roozendaal, Hahn, Nathan, de Quervain & McGaugh, 2004; Roozendaal, Nguyen, Power & McGaugh, 1999; Roozendaal, Okuda, de Quervain & McGaugh, 2006; Roozendaal, Okuda, Van der Zee & McGaugh, 2006). Infusions of atenolol (β-adrenoreceptor antagonist) into the BLA blocks glucocorticoid-mediated memory enhancement when RU28362, a GR agonist, was infused simultaneously (Quirarte, Roozendaal & McGaugh, 1997). Infusions of β-adrenoreceptor antagonists also blocked the memory enhancing effect of systemically delivered dexamethasone post-training (Quirarte, Roozendaal & McGaugh, 1997), further suggesting the necessity of β-adrenergic activation for glucocorticoid-induced memory consolidation in the BLA. Recent evidence also suggests cholinergic activation of the BLA may be required for

glucocorticoid-induced modulation of memory consolidation (Power, Roozendaal & McGaugh, 2000).

Studies of stress hormone effects in human subjects have generally been consistent with those of animal fear conditioning models. Antagonism of the β-adrenoreceptor prevents memory consolidation of an emotionally arousing event (Cahill, Prins, Weber & McGaugh, 1994; van Stegeren, Everaerd, Cahill, McGaugh & Gooren, 1998), and reduces the prevalence of PTSD symptoms when administered following an acute traumatic event (Pitman, Saunders, Zusman, Healy, Cheema, Lasko et al, 2002; Vaiva, Ducrocq, Jezequel, Averland, Lestavel, Brunet et al, 2003). In addition, cortisol, the human analog of corticosterone, appears to enhance memory for emotionally arousing material in human subjects (Buchanan & Lovallo, 2001).

1.4. Benzodiazepine Treatment of PTSD

Benzodiazepines have been widely used in the treatment of anxiety, and act at inhibitory $GABA_A$ receptors to produce their anxiolytic effect. However, benzodiazepine drugs appear to have little or no prophylactic efficacy when used to treat PTSD patients (Davidson, 1997; Gelpin, Bonne, Peri, Brandes & Shalev, 1996; Taylor & Cahill, 2002). Under the assumption that predator stress models aspects of PTSD, treatment with a $GABA_A$ receptor agonist should have no prophylactic effect when tested in this model.

1.5. General Comments

Both predator stress and conditioned fear models have shown common mechanisms of neural plasticity involved in the consolidation of memory for an emotionally arousing stimulus. As in fear conditioning, anxiety-like behavior following predator stress is

NMDA receptor dependent (Blundell, Adamec & Burton, 2005; Adamec, Burton, Shallow & Budgell, 1999a), and induces right hemispheric LTP of amygdala afferent and efferent pathways (Adamec, Blundell & Collins, 2001; Adamec, Blundell & Burton, 2005a). This parallels findings of lateralized amygdala hyperexcitability in humans suffering from PTSD (Rauch, Whalen, Shin, McInerney, Macklin, Lasko et al, 2000; Shin, Wright, Cannistraro, Wedig, McMullin, Martis et al, 2005). Furthermore, exposure to a predator increases plasma levels of corticosterone and ACTH (Adamec, Kent, Anisman, Shallow & Merali, 1998), and results in protein synthesis dependent changes in affect resulting from the activation of GRs by corticosterone (Adamec, Strasser, Blundell, Burton & McKay, 2006).

Given these many parallels, it is of clinical and scientific interest to determine the role of corticosteroid and noradrenergic receptors in the lasting effects of predator stress on affective behavior. To address this issue, we investigated the effect of single post-exposure systemic injections of either RU486 (GR antagonist), propranolol (β-adrenoreceptor antagonist), or a combined injection of RU486 and propranolol on anxiety-like behavior. Reduction in levels of anxiety-like behavior following predator stress from such injections would both replicate the findings of fear conditioning models, and pharmacologically validate predator stress as an animal model of PTSD. The effect of post-stress injections of spironolactone (MR antagonist) and chlordiazepoxide (GABA agonist) were also investigated.

2. Methods

2.1. General Comments on Methods

This thesis represents the combination of two studies. Initially, the prophylactic effects of a β-adrenergic and GR blocker were investigated. The effects of the GABA agonist chlordiazepoxide on anxiety-like behavior were also examined. At a later date, a second study was conducted using novel groups to build on the findings of the first. The methodology of the two studies is detailed below, and where possible the combined results of both studies will be presented.

2.2. Animals

Two hundred eighty male Long Evans rats (Charles River Canada) were used. All rats weighed between 110g and 120g on arrival, and were housed individually in standard clear polycarbonate cages. The animals were fed ad lib, and were maintained on a 12 hour light-dark schedule (lights on at 7 a.m.). They were given a one day acclimatization period to their home cage, after which they were handled once per day for one minute over the following three days. Handling consisted of lifting the rat with a gloved hand while supporting it using the opposite forearm. The animals were then lightly stroked until the one minute had elapsed, at which time they were returned to their home cages. It must be noted that in study two, rats were handled five times over five days as opposed to the three times reported for study one.

2.3. Groups

All rats were randomly assigned to experimental groups. In the first study, animals (160 rats) were randomly assigned to eight groups of twenty rats. On the experimental day, groups were treated as follows: predator stressed only (exposed to a cat with no injection), predator stressed vehicle-post (exposed to a cat and then vehicle-injected),

predator stressed vehicle-pre (vehicle-injected 30 minutes prior to being exposed to a cat), predator stressed propranolol-post (exposed to a cat and then injected with propranolol), predator stressed propranolol-pre (injected with propranolol 30 minutes prior to being exposed to a cat), predator stressed chlordiazepoxide-post (exposed to a cat and then injected with chlordiazepoxide), and predator stressed mifepristone-post (exposed to a cat and then injected with RU486). There was also a handled control group in which rats were not exposed to a cat at any time.

Several months later, in the second study, animals (120 rats) were randomly assigned to six groups of twenty rats. Groups consisted of four predator stressed groups: vehicle injection post-exposure, spironolactone injection post-exposure, propranolol injection post-exposure, and combined RU486 + propranolol injection post-exposure. There were also two handled control groups: non-injected control and vehicle injection following handling.

2.4. Drug Administration

For study 1 (Table 1), the doses were: chlordiazepoxide (10 mg/kg), RU486 (20 mg/kg), and propranolol (5 mg/kg). All doses were suspended and sonicated in 1mL Tween 80 vehicle solution (prepared by adding two drops Tween 80 to 10mL of sterile saline and sonicating for 10 minutes). Chlordiazepoxide dosage was chosen to be within a behaviorally effective and anxiolytic range when tested 30 minutes after administration (File, Lister & Nutt, 1982; Kennet, Bright, Trail, Baxter & Blackburn, 1996). The dose of RU486 has previously been demonstrated to produce effective blockage of stress effects on limbic physiology (Xu, Holscher, Anwyl, & Rowan, 1998), while the dosage of

propranolol has been shown to interrupt the consolidation of fearful memories when administered following a traumatic, stressful event (Sternberg, Korol, Novack, & McGaugh, 1986). All drugs were prepared fresh daily and just before use. Pre-exposure injections of propranolol occurred 30 minutes before predator stress, while all other injections were administered 1 minute after being exposed to a cat.

In study 2 (Table 2), the doses were: propranolol (10 mg/kg), RU486 + propranolol (20 mg/kg RU486 and 5 mg/kg propranolol), and the MR blocker, spironolactone (50 mg/kg). The 10 mg/kg dose of propranolol was administered to examine dosage effects, while the spironolactone dose is known to be effective and well tolerated, with little effect on spontaneous behavior (Koenig & Olive, 2004). All doses were suspended and sonicated in Tween 80 as described previously. All injections were administered 1 minute after exposure. There were no pre-exposure injections.

2.5. Treatment

For both studies, rats were tested in batches over a ten week period. In the first study, batches consisted of 16 rats, two from each of the eight groups. In the later study, batches contained 12 rats, also composed of two animals from each of the six novel groups. Because equal numbers of representatives from each group were tested together each week, possible extraneous sources of variability from batch effects were controlled.

2.5.1. Predator Stress and Handling

One week after arriving in the lab, animals in the predator stress groups were exposed to one of two cats. Exposures were unprotected and occurred between the hours of 9 a.m. and 1 p.m. Cat used and time of exposure were counterbalanced in all groups to ensure a

similar exposure experience for all rats in the different groups. All exposures took place in a large enclosed room with a floor area of approximately 35 square feet. Cats were placed in the room prior to rat entry by way of a standard door. Rats were then brought to the room in a small gray opaque box. This box was fitted to match a small trap door in the wall of the cat room, allowing the rat to be placed into the cat room without handling by the experimenter, and without distracting the cat. Exact specifics of this method have been described in great detail elsewhere (Adamec & Shallow, 1993). Exposures lasted for ten minutes, beginning once the rat was ejected from the box into the cat room. The ten minute exposure was videotaped to capture the activities of both the rat and the cat.

Cat response to the rat generally consisted of the cat watching the rat from a distance, followed by several approaches, pawing, and the occasional mild attack. Although given unrestricted access to the rat, the cats did not injure the rats in any way. Rats were examined for physical injuries following exposure, and consistently none were observed.

On the day of cat exposure for the predator stressed groups, the rats in the handled groups were handled in the method described previously. These rats were also housed separately, and at no time did they come into contact with predator stressed animals.

Time of treatment was counterbalanced among all groups. Following treatment, all rats were returned to their home cages and left unhandled until behavioral testing.

2.5.2. Behavioral Measures taken from Cat Exposures

Behavior of both the rat and cat was analyzed from videotape to produce a measure of the cat exposure experience within each of the experimental groups.

2.5.2.1. Cat Measures

Cat behaviors analyzed included latency to approach the rat, the number of approaches, and time spent near the rat. Latency to sniff the rat and the time spent sniffing were also scored, as was the latency to bite the rat, number of bites, and frequency of pawing. The floor of the exposure room was divided into one foot squares using masking tape. The cat was considered to be near the rat when it moved to within one foot of it.

2.5.2.2. Rat Measures

Rat behavior in response to the cat was also analyzed. Defensive behavior was categorized as either active, passive, or escape. Active defense included rat initiated approaching, biting, and pushing of the cat with a forepaw. Passive behavior was characterized by freezing (duration greater than one second) when the cat was near the rat. Escape behavior was defined as rapid movement away from the cat in response to an approach. Overall time spent immobile was also measured. The frequency of each of these behaviors was analyzed from videotape.

2.6. Post-Treatment Behavioral Testing

Anxiety like behavior was examined in the light-dark box, social interaction test, hole board, and elevated plus maze at least one week after cat exposure. Such tests are commonly used to assess rodent exploration, activity, and anxiety (File, 1987; File & Wardhill, 1975a; File & Wardhill; 1975b). Video recording equipment was used to record all behavioral tests, which were analyzed blind at a later time. All tests were 5 minutes in duration and conducted between the hours of 8 a.m. and 1 p.m.

2.6.1. Light-Dark Box

Seven days after treatment, rats were tested in the light-dark box. The box was divided into two chambers of equal size, and both halves were covered with transparent plastic covers. The walls of the light chamber were painted white, and its floor was wooden. The corresponding dark chamber had black walls and a mesh metal grating on the floor. The transparent top of the dark chamber was covered to prevent illumination from an overhead light source. Testing occurred in a darkened room, lit only by a 100 watt bulb positioned directly over the light chamber. Light intensity measured on the floor of the light chamber was 850 lx, and 0 lx on the floor of the dark chamber. Two light-dark boxes were constructed to allow for the simultaneous testing of two animals at one time. The boxes were constructed from 2.5 cm thick plywood, and each chamber measured 32 x 10.5 x 14 cm (length x width x height).

Animals were placed into the light chamber facing away from the darkened chamber, and were allowed to explore both sides freely for 5 minutes. As with cat exposures, tests were videotaped, and analyzed at a later time to avoid any effect of an experimenters' presence. Following each test boxes were cleaned with a 5% alcohol solution and wiped dry.

Measures taken from the light-dark box included: latency to enter the dark chamber, time spent in each chamber, and the frequency of entry into each side of the box. Rats were considered to be in a chamber when all four feet were within the boundaries of that chamber.

2.6.2. Social Interaction

Following the testing of all rats in the light/dark box (approximately 90 minutes elapsed), rats were tested in the social interaction test. The test took place in an open top square wooden box measuring 60 x 60 x 35 cm (length x width x height). The walls were painted black, while the floor remained unpainted. Testing occurred in a darkened room, lit by a red incandescent bulb.

For each test, animals were placed into the box in pairs, and allowed to interact for 5 minutes. Tests were video recorded, and analyzed at a later time. For identification purposes, one rat in each pair had its back darkened using a non-toxic black marker. Rat partners were from different groups, and therefore rat-marking and group-pairing was counterbalanced between groups. This provided a degree of control over any possible stress experienced during the marking procedure, or from the different group pairings. Following each social interaction test, rats were returned to their home cages and the box was cleaned using a 5% alcohol solution and wiped dry. Testing for a given rat then ceased for that day.

Measures taken from the social interaction test included the time spent interacting for each rat in the pair, the number of pursuits (following a partner immediately after it withdraws), and the frequency of fights. Rats were considered to be socially interacting when they were in close proximity to, and facing the test partner.

2.6.3. Hole Board

On the day following testing in the light/dark box and social interaction test, animals were tested in the hole board followed by testing in the elevated plus maze (see 2.6.4.). The hole board test is used to provide independent measures of activity and exploratory

tendency (File & Wardhill, 1975). The hole board apparatus was constructed with the same dimensions as those used for the social interaction test box, except that four evenly spaced holes were drilled 14 cm from the walls in a floor that was raised 12 cm above the ground. Both floor and walls were painted with grey enamel. Using black electrical tape, a small square was created inside the box, separating it into center (containing the 4 holes) and perimeter (near the wall) segments. During testing in the hole board, the room was lit normally.

Each rat was placed in the center of the box using a gloved hand and was allowed to travel freely for 5 minutes. Rats were then immediately transferred to the elevated plus maze for a further 5 minutes of testing. Following each test, the box was cleaned with a 5% alcohol solution as before. As with the other behavioral tests, the hole board was video recorded and analyzed blind at a later time.

For each hole board test, activity and exploratory behavior was measured. Activity was recorded as the number of rears and time spent in motion of any kind. Exploratory tendency was scored as the number of head dips (placing entire head into one of the four holes drilled in the floor), as well as the amount of time spent in the center, and near the walls of the box. Rats were considered to be in either the center or near the wall of the box when all four feet were respectively inside or outside the square created using black electrical tape.

2.6.4. Elevated Plus Maze (EPM)

Following testing in the hole board, rats were transferred using a gloved hand to the elevated plus maze. The EPM consisted of a wooden four-armed platform with arms

arranged in the shape of a plus. The apparatus was painted with gray enamel, and was raised 50 cm above the floor. Arms were 10 cm in width and 50 cm in length, two of which were protected while the two remaining arms (arranged perpendicularly to the first) remained open. Protected arms were surrounded by 40 cm walls which were open at the top, while the remaining two arms were surrounded only by a small lip (3 cm high) which indicated to the animal that it was near the edge. The four arms intersected to form a square center platform with an area of 100 cm^2 (10 x 10 cm). As with the hole board, tests in the EPM were conducted under normal lighting conditions.

At the start of each test, rats were placed in the center square facing the same open arm, and were allowed to move freely for 5 minutes. All tests were video recorded and analyzed blind at a later time. At the conclusion of each test, the maze was cleaned and wiped dry using a 5% alcohol solution. Rats were then immediately returned to their home cage, and behavioral testing for that animal was complete.

A number of behavioral measures were analyzed from the EPM. These included a standard measure of anxiety-like behavior, and several additional assessments of open arm exploration. The standard measure of anxiety was ratio time; defined as total time spent in the open arms of the maze divided by the total time spent in any arm of the maze. Smaller ratios in this measure indicate less open arm exploration, and the rat is assumed to be more "anxious".

Additional measures involved the analysis of head dips, which were categorized as either protected, unprotected, or center, based on the position of the animals hindquarters at time of head dip. Protected head dips were scored when a rat dipped its head over the

side of an open arm with its hindquarters in the closed arm of the maze. Unprotected head dips occurred when all four feet were contained in the open arm of the maze. Center dips were scored when an animal dipped its head and had all four feet securely placed inside the center square of the maze. Protected, unprotected, and center frequencies of rearing were also recorded in a similar fashion.

Finally, risk assessment was measured. Risk assessment was scored when a rat poked its head into an open arm of the maze while its hindquarters remained safely in one of the closed arms. The frequency of these assessments was recorded, as was the time spent engaged in risk assessment. To produce a relative frequency risk assessment measure, the time spent in risk assessment was divided by the total time spent in the closed arms of the maze.

2.7. Acoustic Startle Testing

Testing in the startle chamber was conducted following the completion of behavioral testing. Due to the large number of animals in each batch, it was not possible to test all animals in the startle chamber on the same day. Therefore, half of the animals in each batch were tested on day 8 post-exposure, with the remaining half were tested on post-exposure day 9. Date of startle testing was counterbalanced between groups to ensure equal numbers from each group were tested on each day.

Unconditioned startle response to an acoustic stimulus was determined using a standard startle chamber (San Diego Instruments). The apparatus was fitted with a plastic cylinder (20.3 cm in diameter) which was used to hold the animal, as well as a speaker for producing sound bursts. A piezoelectric transducer positioned directly below the

cylinder was used to record the motion of a rat during each sound burst. Output from the transducer was led to a computer for sampling.

Prior to startle testing, animals were acclimatized to the apparatus for 5 minutes with a background white noise level of 60 db. Following acclimation, rats received a 50 millisecond burst of 120 db rising out of the 60 db background noise once every 30 seconds for 20 minutes. Of the 40 trials, 20 were conducted in the light, and the remaining 20 in the dark. Light and dark trials were presented randomly by way of a computer, and all inter-trial intervals were spent in the dark. For a light trial, lights came on 2.95 seconds before the acoustic sound burst was delivered, and remained on for the 50 millisecond burst. Thus, the total time a light was on equaled 3 seconds. At the conclusion of the 3 second light period, the light was extinguished. Light intensity was 301 lx.

A computer attached to the apparatus recorded 40 samples of transducer output. Samples included a 5 millisecond baseline and 250 millisecond sample after onset of the noise burst. Average transducer output just prior to the noise burst was saved as a baseline (V_{start}). In addition, the computer determined the peak startle amplitude within each of the samples (V_{max}) and this value was also stored for later analysis. Peak startle amplitude was expressed as $V_{max} - V_{start}$ for analysis. At the end of each startle session the rats were returned to their home cages, and the apparatus was washed using warm water.

2.8. Statistical Analysis

All data were tested for normality using the D'Agostino Omnibus Test. When deviations from normality were substantial ($p<.01$), the Kruskal-Wallis one-way non-parametric analysis of variance on medians was used. Details appear in results. Planned comparisons were conducted using Fischer's LSD or Kruskal Wallis multiple comparison Z test when appropriate. Differences were considered significant if $p \leq .05$.

2.9. Ethical Approval

The research methods used in all experiments were reviewed for compliance with the guidelines of the Canadian Council on Animal Care (CACC), and approved by the Institutional Animal Care Committee of Memorial University. All efforts were made to minimize pain, stress, and the number of animals used.

3. Results

3.1. Consistency of the Predator Stress Experience

In both studies, neither cat nor rat behavior measured during cat exposures showed any behavioral differences between exposed groups (all $F\{10,209\} \leq 1.48$, $p>0.14$). As such, cat approach-attack behavior and rat reaction to the cat can be considered equivalent across groups.

3.2. General Comment on Groupings

As reported in the methods, this thesis represents the combination of work completed at different times. For clarity, it was of interest to compare all drug groups in combined analyses where appropriate. Statistically, this proved possible for all test results except for response to startle. Unanticipated vehicle effects on startle response in some cat

exposed animals, prevented collapse of data across groups. Therefore, results of animals in each drug condition to acoustic startle are presented separately and last.

Cat exposed and cat exposed given vehicle groups did not differ in tests of anxiety-like behavior (except startle), nor did handled and handled given vehicle groups. Therefore, the groups were combined into new comparison groups: combined handled control and combined predator stress. These groups were then used in subsequent comparisons with the remaining predator stressed given drug groups.

In analyzing drug effects, the results are reported in 3 separate groupings. First, comparisons between all propranolol injected animals are analyzed together. A second analysis examines the effects of GR and MR blockers, as well as a combined RU486 + propranolol injection on anxiety-like behavior. Finally, the effects of the benzodiazepine receptor agonist chlordiazepoxide are assessed.

3.3. Effect of β-Adrenoreceptor Block on Behavior Following Exposure to a Predator

Five groups were compared: 5 mg/kg propranolol pre stress (administered 30 minutes before cat exposure), 5 mg/kg propranolol post stress (administered 1 minute after exposure), 10 mg/kg propranolol post stress (administered 1 minute after exposure), combined handled control, and combined predator stressed,.

3.3.1. Light-Dark Box

Exposure to a predator significantly reduced the frequency of entries into the light chamber, while increasing mean time spent in the dark chamber of the box (all $F\{4,195\} \geq 4.80$, $p<.002$; mean contrasts, $p<.05$; Figure 1, top panel). Low dose propranolol (5 mg/kg), given either before or after cat exposure did not reduce the stress-

induced increases in anxiety in the light dark box. However, administration of a 10 mg/kg dose post stress returned time spent in the dark and entries into the light to control levels (Figure 1, top panel).

Predator stress also reduced the latency to enter the dark chamber of the box ($\chi^2\{4\}=12.21, p<.02$; median contrasts, $p<.05$). Low dose propranolol had no anxiolytic effect on latency. A larger propranolol dose of 10 kg/mg was also without effect on latency, although a trend toward control levels was present (Figure 1, top panel).

3.3.2. Social Interaction Test

Exposure to a predator also significantly reduced time spent interacting. Only the 10 mg/kg post stress injection of propranolol was effective in returning interaction to control levels ($F\{4,195\}=12.15, p<.001$; mean contrasts, $p<.05$). The same pattern was observed in the analysis of fighting behavior ($\chi^2\{4\}\geq 17.21, p<.002$; median contrasts, $p<.05$). Predator stress reduced the frequency of fighting in the social interaction test, which was reversed to control levels after post-stress treatment with 10 mg/kg propranolol (Figure 1, middle panel).

Groups again differed in pursuit behavior ($\chi^2\{4\}\geq 17.21, p<.002$; median contrasts, $p<.05$). Animals made fewer pursuits following exposure to a predator, and this suppression was not alleviated by the propranolol injections (Figure 1, middle panel).

3.3.3. Hole Board

There were group differences in the amount of time spent near the wall in the hole board, with cat exposed animals being significantly more thigmotaxic than handled controls ($F\{4,195\}=3.96, p<.005$; mean contrasts, $p<.05$). As in other tests, the 10 mg/kg

dose of propranolol was effective in returning time spent near the wall to control levels, while injection of low dose propranolol was not (Tukey Kramer test, $p<.05$; Figure 1, bottom panel).

Measures of activity and exploration in the hole board were not affected by predator stress, which has been a common observation of past studies using this model (Adamec, 1997). Moreover, there were no drug effects on these measures. As such, this result will not be reported in subsequent sections to avoid redundancy.

3.3.4. Elevated Plus Maze

Group differences were observed in both the ratio-time and ratio-frequency risk measures of the EPM (all $\chi^2\{4\} \geq 11.79$, $p<.019$; median contrasts, $p<.05$). Exposure to a predator reduced median ratios in both measures. This decrease was not affected by low dose propranolol (given either before or after exposure), whereas 10 mg/kg propranolol treatment post stress restored ratios to the level of handled controls (Figure 1, bottom panel).

As in the hole board, there were no group differences in measures of activity and exploration in the EPM. Similarly, activity and exploration in the EPM was not affected following propranolol injection, or any other drug treatment condition. When considered in conjunction with results from the hole board, absence of activity and exploration differences suggest that group differences are a result of defensive or anxiety-like behavior, and not exploratory tendencies toward a novel environment.

3.4. Effect of GR and MR Block on Behavior Following Exposure to a Predator

Six groups were compared: combined handled control, combined predator stress, 20 mg/kg RU486 post stress, 50 mg/kg spironolactone post stress, 5 mg/kg propranolol post stress (carried over from results section 3.3.), and 20 mg/kg RU486 + 5 mg/kg propranolol post stress.

3.4.1. Light-Dark Box

As reported above, cat exposed animals entered the light chamber less frequently and spent more time in the dark chamber of the box than did controls (all $F\{5,219\} \geq 2.82$, $p<.02$; mean contrasts, $p<.05$). Low dose propranolol treatment after predator stress had no effect on stress-induced changes in these measures (Figure 1, top panel; Figure 2, top panel). In comparison, the GR antagonist RU486 showed a trend toward returning time spent in the dark back to the level of handled controls. A combined administration of RU486 + propranolol also partially blocked the effects of predator stress on time spent in the dark. This combined treatment also partially blocked the stress-induced suppression of entries into the light chamber following cat exposure. Finally, MR block using spironolactone had no effect on predator stress effects in the light-dark box (Figure 2, top panel).

Only treatment using propranolol + RU486 in combination post stress was effective in returning the stress-induced reduction in latency to enter the dark chamber to baseline ($\chi^2\{4\}=14.35$, $p<.02$; median contrasts, $p<.05$). Injections of RU486, spironolactone, or 5 mg/kg propranolol given singly were ineffective in blocking the effect of stress on latency (Figure 2, top panel).

3.4.2. Social Interaction Test

Predator stress significantly reduced time spent interacting. This effect was reversed by the administration of spironolactone, and also by combined treatment of RU486 and propranolol ($F\{5,214\}=12.26$, $p<.001$; mean contrasts, $p<.05$). Neither injections of RU486 nor 5 mg/kg propranolol given alone were effective in returning time spent interacting to control levels (Figure 2, middle panel).

Group differences were also observed in pursuit behavior and fighting frequency (all $\chi^2\{5\} \geq 14.64$, $p<.02$, median contrasts, $p<.05$). As previously reported, cat exposure significantly reduced the frequency of pursuits and fights in the social interaction test. Injection of the MR receptor blocker spironolactone was effective in blocking the exposure-dependent decrease in pursuit behavior, but did not reverse stress induced suppression of fighting. All other treatments were without effect on pursuit and fighting frequency, although a trend toward control levels for pursuit behavior was seen following treatment with RU486 or 5 mg/kg propranolol. Interestingly, combined injection of RU486 and propranolol was without effect on predator stress induced suppression of pursuits (Figure 2, middle panel).

3.4.3. Hole Board

Post stress administration of RU486 or 5 mg/kg propranolol alone failed to prevent stress induced increases in time spent near the wall in the hole board ($F\{5,214\} = 8.31$, $p<.001$; Tukey-Kramer, $p<.05$). However, treatment with ineffective doses of RU486 and propranolol in combination effectively eliminated stress-induced increases in time spent near the wall, as did injection of the MR blocker spironolactone (Figure 2, bottom panel).

3.4.4. Elevated Plus Maze

As reported in earlier sections, exposure to a predator significantly reduced median ratio time and median ratio risk in the EPM (all $\chi^2\{5\} \geq 12.36$, $p<.032$; median contrasts, $p<.05$). Post stress administration of the GR blocker RU486 alone failed to prevent stress induced reduction of median ratios, as did post-stress injection of 5 mg/kg propranolol. However, treatment with ineffective doses of propranolol and RU486 in combination was effective in blocking the effects of predator stress on behavior in the EPM. The MR blocker spironolactone also blocked predator stress effects on ratio time and ratio risk (Figure 2, bottom panel).

3.5. *Effect of Chlordiazepoxide Treatment on Behavior Following Exposure to a Predator*

The injection of 10 mg/kg chlordiazepoxide one minute after cat exposure was without effect when rats were tested 1 week later for stress-induced changes in behavior. Chlordiazepoxide injected animals were comparable to the combined predator stressed group rats in all behavioral measures of the light-dark box, social interaction test, hole board, and EPM (all $F\{2,157\} \geq 5.52$, $p<.01$; all $\chi^2\{2\} \geq 5.38$, $p<.07$; all mean contrasts, $p<.05$; all median contrasts, $p<.05$). Results from these tests appear in Figure 3.

3.6. Response to Acoustic Startle

Startle data required separate analysis, as vehicle injection resulted in the suppression of startle in study 1, but not in study 2. Therefore, animals used as controls in the first study could not be combined with those from the second, although predator stress alone increased startle in both studies.

Groups were compared with respect to body weight prior to analysis of startle, and there were no group differences at the time of testing. Differences did exist between startle in the light and dark ($F\{1,115\}=20.53$, $p<.001$), but these conditions were combined since the pattern of responses across groups in both conditions did not differ.

3.6.1. Effects of Chlordiazepoxide Post-Stress on Peak Startle Amplitude

Group effects were observed between rats treated with chlordiazepoxide and handled, predator stressed, and predator stressed with vehicle injection groups ($\chi^2\{4\}=48.45$, $p<.001$; median contrasts, $p<.05$). Exposure to a predator resulted in significant increases in peak startle amplitude, but vehicle injection prior to or following exposure suppressed amplitudes below the level of predator stressed only rats and handled controls (Figure 4, upper panel).

Due to the similar responses of pre-exposure and post-exposure vehicle injected groups, they were combined, and used as a single comparison group (stressed + vehicle combined) in subsequent analyses. Finally, treatment with chlordiazepoxide blocked the effect of vehicle injection, and increased peak startle amplitude above the level of vehicle injected predator stressed rats to that of cat exposed only rats (Figure 4, upper panel).

3.6.2. Study 1: Effects of RU486 and Propranolol (5 mg/kg) Pre- and Post-Stress on Peak Startle Amplitude

Cat exposure alone increased peak startle amplitude, while vehicle injection plus cat exposure blocked and suppressed stress-induced increases in peak startle amplitude to a level below both control and cat exposed only groups (all $\chi^2\{3\}\geq 54.38$, $p<.001$; median contrasts, $p<.05$). Suppression of startle was also seen following treatment with 5 mg/kg

propranolol before and after stress (Figure 4, lower panel). In contrast, post stress GR block with RU486 prevented vehicle-induced suppression of startle. Startle amplitude in these rats was elevated above the level of handled controls to one comparable to predator stressed only animals (Figure 4, lower panel).

3.6.3. Study 2: Effects of Propranolol (10 mg/kg), Spironolactone, and Combined (RU486 + Propranolol) Post Stress on Peak Startle Amplitude

In study 2, no difference was observed when vehicle-injected handled were compared to rats in the handled only group. There was also no difference between vehicle-injected exposed animals and rats in the exposed only group. Therefore the groups were combined to form a combined handled and combined predator stressed group for subsequent analyses.

As in study 1, exposure to a predator significantly increased peak startle amplitude ($\chi^2\{4\}=21.43, p<.001$; median contrasts, $p<.05$). Treatment with 10 mg/kg propranolol post stress had no effect on stress-induced enhancement of startle. In contrast, MR block as well as post-stress treatment with RU486 and propranolol (5 mg/kg) in combination blocked stress effects on startle (Figure 5).

3.7. Habituation of Startle Analyses

Predator stress has previously been shown to prolong the habituation to startle (Adamec, 1997), and the time to habituate was therefore compared across groups. Startle amplitude over 20 trials (light and dark combined) were averaged, and a plot of these averages was used to estimate a trial constant (τ) by fitting plots of peak startle amplitude (Jandel Table Curve V4.0) to the exponential decline function:

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

where y and y_0 are peak startle amplitude, a is a constant, e is the base of the natural logarithm, t is trial, and τ (tau) is the trial constant (number of trials required to decline to 37% of the maximal peak startle amplitude). The trial constant is taken as a measure of habituation rate – the greater τ, the greater the delay to habituate. Data were smoothed to improve fit using a curve fitting program, and special care was taken to ensure the smoothing did not distort the data. An example of smoothed fit can be seen in Figure 6. Estimates of τ included a standard error estimate, which was used to calculate t-statistics between the trial constants of different groups. Planned comparisons of the τ estimates for each group were done using two-tailed t-tests.

3.7.1. Study 1: Effects on τ of RU486, Chlorodiazepoxide, and Propranolol (5 mg/kg) Pre- and Post-Stress

As in previous studies, predator stress and predator stress given a vehicle injection delayed habituation to startle (increased τ; Figure 6, middle panel). In comparison, treatment with chlordiazepoxide after stress attenuated the delay of habituation, but did not block it. Values of τ in these rats were elevated over controls, but less than those observed in predator stressed and predator stressed plus vehicle groups. Like chlordiazepoxide, RU486 and propranolol given post-exposure attenuated, but did not block delayed habituation to startle. Habitiuation in these groups took longer than in controls, and had τ values between predator stressed rats and those treated with chlordiazepoxide. Propranolol given prior to exposure was the only drug treatment to reduce delay of habituation to the level of controls. τ values in this group fell between

control animals and those receiving propranolol (or RU486) after cat exposure (all $t\{38\} \geq 2.06$, $p<.046$, Figure 6, middle panel).

3.7.2. Study 2: Effects on τ of Propranolol (10 mg/kg), Spironolactone, and Combined (RU486 + Propranolol) Post-Stress

As in study 1, predator stress significantly increased the delay of habituation to startle (Figure 6, bottom panel). In contrast, propranolol given in the higher dose of 10 mg/kg increased stress-induced delay of habituation. Animals in this group displayed larger τ values than both controls and combined predator stressed groups. On the other hand, animals given the MR blocker spironolactone post stress did not differ from controls in their habituation to startle, suggesting that mineralocorticoid receptors may participate in stress induced delay of habituation. Finally, treatment with a combination of RU486 and 5 mg/kg propranolol post stress resulted in partial reduction of the delay to habituate. These animals habituated more slowly than handled controls, but faster than both combined predator stressed rats and predator stressed rats treated with 10 mg/kg propranolol (all $t\{38\} \geq 2.27$, $p<.028$; Figure 6, bottom panel). This is of interest, as both drugs were found to be ineffective in reducing the delay to habituate when administered singly (Sect. 3.6.1.), yet were effective when given in combination. Such results suggest a possible synergy between the GRs and β-adrenoreceptors in consolidating the delay of habituation to the acoustic startle test following predator stress.

4. Discussion

4.1. General Comments

In the results, it was shown that the predator stress experience did not differ between groups. It was also shown that treatment with vehicle or drug had no effect on activity or exploratory tendency in the hole board and plus maze. As such, group differences in anxiety-like behavior appear to result from pharmacological manipulation, and not variations in cat-rat interactions or activity differences following stress. These findings are consistent with known effects of predator stress, and of neuropharmacological treatment to modify these effects (Adamec & Shallow, 1993; Adamec, Bartoszyk & Burton, 2004).

4.2. Receptor-Mediated Consolidation of the Lasting Changes in Affective Behavior Observed Following Predator Stress

4.2.1. β-adrenoreceptor Involvement

Present findings suggest that β-adrenoreceptors participate in the consolidation of anxiogenic effects of predator stress. Post-stress treatment with propranolol dose dependently reduced or blocked stress effects in all measures of rodent anxiety except for pursuits in the social interaction test (Figure 1). In this instance, low dose propranolol given either before or after predator stress partially blocked suppression of pursuits, whereas post-stress treatment using a 10 mg/kg dose was totally ineffective. Such partial effects are difficult to interpret, as rats in these groups did not differ from predator stressed or handled controls. One possible explanation is that pursuit behavior is mediated by a mechanism separate from other measures of anxiety-like behavior. In fact, previous work in this lab has produced factor analytic data to support this view (Adamec, Blundell & Burton, 2003).

Although propranolol treatment was tested pre- and post-stress, little can be said regarding the efficacy of a 5 mg/kg pre-stress β-adrenoreceptor block. When given after cat exposure, a relatively high dose of 10 mg/kg was required to block the consolidation of anxiogenic stress effects. This dose was not tested pre-stress, and the 5 mg/kg dose used may have been too low to have any anxiolytic properties. Further studies using 10 mg/kg pre-stress propranolol treatment are warranted.

It is of interest that high dose propranolol was required to block anxiety-like behavior following predator stress, as 5 mg/kg treatment has previously been shown to interfere with rodent memory for a stressful learning task (Cahill, Pham & Setlow, 2000). There are several reasons why this may be so. Firstly, norepinephrine release following unprotected exposure to a cat may be considerably greater than that produced using fear learning paradigms. As such, a more robust blockade of β-adrenoreceptors may be required to interfere with the consolidation of stress effects, although this idea remains to be tested. Secondly, evidence has implicated the amygdala in the consolidation of fearful memories (Ferry, Roozendaal & McGaugh, 1999a; Roozendaal, Okuda, de Quervain & McGaugh, 2006) and to the lasting changes in affect observed following predator stress (Adamec, Blundell & Burton, 2005b; Adamec, Burton, Shallow & Budgell, 1999b). Finally, fear learning (Ferry, Roozendaal & McGaugh, 1999b) and exposure to a predator (McIntyre, Kent, Hayley, Merali & Anisman, 1999) increase norepinephrine levels in the rodent amygdala. Moreover, the magnitude of release is directly related to the degree of consolidation of fear memory (Roozendaal, Okuda, de Quervain & McGaugh, 2006).

In addition, fear memory consolidation is facilitated through co-activation of β-adrenoreceptors and GRs in the amygdala (Roozendaal, Okuda, de Quervain & McGaugh, 2006). Assuming a similar process occurs following predator stress, surges of corticosterone during and after exposure to a cat may facilitate β-adrenoreceptor mediated consolidation of stress effects. Consistent with this suggestion, strong and long lasting surges of plasma corticosterone in rats were found following exposure to a predator (Adamec, Blundell & Burton, 2006).

Present data lend further support to a role of β-adrenoreceptors and GRs in the consolidation of memory for an emotional event. Corticosterone and norepinephrine likely regulate the change in affective behavior following stress both individually and cooperatively, evidence for this is provided in the following section.

4.2.2. GR and MR Involvement

The present data suggest that GRs work in concert with β-adrenoreceptors in the consolidation of stress-effects in memory following exposure to a cat. Neither GR block nor antagonism of the β-adrenoreceptor alone altered the sensitizing effects of predator stress on most measures of rodent anxiety. However, when administered together, the two previously ineffective treatments interfered with consolidation of anxiety-like behavior in the light-dark box, social interaction test, hole board, and elevated plus maze (Figure 2).

There are of course, exceptions, and the pattern varies with the test and behavioral measure. In the light-dark box, GR block alone or in combination with propranolol (5 mg/kg) both partially returned time spent in the dark chamber to control levels. In this instance, stress-effects may result from GR activation alone. It would be of interest to

examine whether higher doses of the GR blocker RU486 could block stress effects completely. In contrast, stress-induced suppression of fighting in the social interaction test was not affected by treatment with RU486 alone, or in combination with the β-adrenoreceptor antagonist propranolol. This suggests that GRs are not involved in stress effects on fighting. However, β-adrenoreceptors appear to have some involvement in mediating this behavior, as post-stress treatment of 10 mg/kg propranolol effectively blocked stress-induced changes (Figure 1). Finally, treatment with RU486 alone or in combination with propranolol was also without effect on pursuit behavior in the social interaction test. Stress-induced suppression of pursuit was partially alleviated by treatment with 5 mg/kg propranolol, suggesting an isolated role of the β-adrenoreceptor in the regulation of this behavior as well.

In a surprise finding, MR block 1 minute after stress prevented the consolidation of a majority of anxiogenic changes normally observed following exposure to a predator. As with propranolol and RU486, anxiolytic efficacy varied with respect to behavioral measure and test. Stress effects were completely blocked in all measures of the hole board and plus maze, as well as time spent interacting in the social interaction test (Figure 2). As previously reported, anxious behavior in these tests was also blocked following combined treatment with RU486 and propranolol, suggesting that the anxiolytic effects of spironolactone and combined treatment may act by way of a common mechanism. However, MR block was also implicated in several GR-independent measures of rodent anxiety. For example, spironolactone partially blocked stress-induced suppression of fighting in the social interaction test, and eliminated stress effects on pursuit behavior

(Figure 2). Present results have also implicated β-adrenoreceptor activation in these measures, while treatment with RU486 was without effect. Finally, MR block failed to prevent stress effects in the light-dark box; these effects appear to depend on concurrent β-adrenergic and GR activation (Figure 2).

To summarize, results suggest that differing mechanisms - perhaps acting in different circuitry - act to consolidate different behavioral changes resulting from traumatic experiences such as exposure to a predator.

4.2.3. Benzodiazepine Receptor Involvement

Treatment with chlordiazepoxide 1 minute after predator stress was without effect on changes in affective behavior (other than startle) observed in rodent tests of anxiety measured 1 week later. Such findings are not unexpected given the data implicating NMDA-dependent LTP in the development of anxiety-like behavior following predator stress (Adamec, Blundell & Burton, 2005a; Adamec, Blundell, & Burton, 2005b). A post-stress increase in GABA mediated inhibition produced by a benzodiazepine anxiolytic would not be expected to interfere with NMDA-receptor dependent LTP already initiated by the stress experience.

4.3. Receptor-Mediated Changes in Startle Following Predator Stress

4.3.1. Effect of Handling on Peak Startle Amplitude

Differences in vehicle effects between studies 1 and 2 complicated the analysis of different receptor systems on startle response following predator stress. Although the direct cause is uncertain, differences may have arisen from the differential handling history of rats in the two studies. In study 1, rats were handled 3 times, whereas in study

2 they were handled five. Consequently, vehicle injection following stress reduced startle amplitude in study 1 (Figure 4), conflicting with previous results which would predict an increase (Adamec, 1997; Adamec, Bartoszyk & Burton, 2004). In study 2, predator stressed rats with and without vehicle injection showed the expected increase in peak startle amplitude (Figure 5), as did exposed only rats in study 1 (Figure 4). Although unexpected, suppression of startle following vehicle injection 1 min after stress is not without precedent (Adamec, Strasser, Blundell, Burton & McKay, 2006). As such, the effect of handling on startle amplitude requires further investigation by way of studies designed to address this issue.

It is possible that increased handling reduced stress responsiveness, whereby rats in study 1 (handled only 3 times) reacted with heightened anxiety to predator stress plus vehicle injection compared to study 2 animals. Accordingly, highly stressed rats may freeze in response to acoustic stress which would interfere with the expected enhancement of startle. Post-stress injection of the anxiolytic benzodiazepine antagonist chlordiazepoxide supports this view, as it blocks the stress plus vehicle induced suppression of startle (Figure 4).

This conclusion applies only to startle reactivity; it does not explain other measures of rodent anxiety. The differential effects of predator stress plus vehicle are consistent with a body of evidence suggesting separable neural substrates are involved in mediating the effects of predator stress on startle and other behavioral tests (Adamec, 2001; Adamec, Burton, Shallow & Budgell, 1999b).

4.3.2. Study 1: β-adrenoreceptor, GR, and Benzodiazepine Receptor Involvement in Startle Suppression and Habituation

RU486 administered post stress effectively prevented the stress plus vehicle induced suppression of startle, replicating previous findings (Adamec, Strasser, Blundell, Burton & McKay, 2006). It is known that plasma corticosterone is elevated 10-fold following predator stress. Past and present results would therefore suggest that startle suppression is a fear reaction precipitated by combined predator and injection stress-induced surges of corticosterone and GR binding. Propranolol, given before or after stress, was without effect on the suppression of startle observed following stress plus vehicle injection (Figure 4). It is possible that the dose tested (5 mg/kg) may have been too low, as was suggested for other tests.

On the other hand, activation of the β-adrenoreceptor appears to be necessary for the initiation of stress-induced delays of habituation (Figure 6, middle panel). This is evidenced by treatment using low dose propranolol post-stress, which returned elevated trial constant values to the level of handled controls (Figure 4). Chlordiazepoxide also reduced the delay to habituate to startle, while GR block with RU486 had no effect (Figure 6).

Taken together, these findings implicate different receptors in the suppression of peak startle amplitude and startle habituation. Stress effects on startle are likely mediated by several mechanisms, and a similar suggestion has recently been made based on electrophysiological data (Adamec, Blundell & Burton, 2005b).

4.3.3. Study 2: β-adrenoreceptor and GR Involvement in Startle Enhancement and Habituation

As previously stated, predator stressed and predator stressed given vehicle groups showed equal enhancement of peak startle amplitude in study 2 (Figure 5). As with low dose propranolol, treatment with a 10 mg/kg dose had no effect on stress-induced facilitation of startle. These data argue against β-adrenoreceptor involvement in the enhancement of peak startle amplitude following predator stress. However, low dose (5 mg/kg) propranolol in combination with RU486 completely blocked startle enhancement (Figure 5). As such, it is premature to deny an involvement of β-adrenoreceptors, as they may act synergistically with GRs in the stress-induced enhancement of peak startle amplitude. Data from study 1 support this conclusion, as RU486 blocked suppression of startle, but was unable to prevent stress-induced startle enhancement (Figure 4). This suggests a codependence between β-adrenoreceptors and GRs in the consolidation of stress effects on peak startle amplitude.

Delay of habituation to startle following stress also appears to depend on the co-activation of β-adrenergic and glucocorticoid receptors. Both propranolol and RU486 were without effect on τ when administered singly in study 1. However, when given in combination, a partial reduction in the delay to habituate was observed (Figure 6). Moreover, high dose propranolol treatment further increased delay of habituation in study 2 (Figure 6, bottom panel).

Stress induced delay in habituation to startle is associated with lasting potentiation of efferent transmission in ACE (Adamec, Blundell & Burton, 2005b, Adamec, Blundell &

Burton, 2003). Additionally, low dose propranolol (4 mg/kg) has been shown to suppress spontaneous neural activity in this area, whereas a 10 mg/kg dose increased it (Simson, Naylor, Gibson, Schneider & Levin, 2001). Therefore, antagonism of β-adrenoreceptors at 10 mg/kg may have increased the delay of habituation to startle by potentiation of efferent transmission and facilitating excitation of the ACE. Conversely, a 5 mg/kg dose may have reduced the delay to habituate by reducing excitation of ACE and subsequent efferent transmission (Figure 6). Central nucleus also contains MRs (McEwen, Weiss & Schwartz, 1968), which is of interest as they are implicated by the present data in the stress induced delay of habituation. Spironolactone successfully blocked both the stress-induced enhancement of peak startle amplitude (Figure 5), and the delay of startle habituation (Figure 6).

4.3.4. Considering Study 1 and 2 Together – The Big Picture

Results of the two studies suggest different mechanisms are engaged in the stress-induced suppression and enhancement of startle. First, chlordiazepoxide was shown to block startle suppression, but was without effect on the stress-induced enhancement of startle amplitude (Figure 4). Second, GR block interfered with startle suppression, but not the enhancement of acoustic startle (Figure 4). Returning to chlordiazepoxide, its lack of effect on startle enhancement is consistent with the view that NMDA-receptor dependent LTP mediates stress effects on behavior. In fact, local block of NMDA receptors in the rodent amygdala prevents startle enhancement, as does a systemic block administered before cat exposure (Adamec, Burton, Shallow & Budgell, 1999a; Adamec, Burton,

Shallow & Budgell, 1999b). Moreover, the block of stress-induced suppression of startle by chlordiazepoxide confirms that it is not an NMDA receptor dependent process.

4.4. Implications in Understanding Neural Mechanisms Involved in the Consolidation of Predator Stress Effects

The pattern of findings in this study is consistent with previous work showing that the impact of predator stress on anxiety-like behavior is mediated by many different substrates (Adamec, 2001; Adamec, Burton, Shallow & Budgell, 1999b). However, it is unknown whether stress effects work on common neuroanatomical areas, or in very different neural circuitry. Present findings of heterogeneous receptor involvement could fit either model. A central locus of action likely exists in the amygdala and hippocampus, as GRs (McEwen, Weiss & Schwartz, 1968; de Kloet, Joels & Holsboer, 2005), MRs (de Kloet, Joels & Holsboer, 2005), and β-adrenoreceptors (Andreasen & Lambert, 1991; Watanabe, Ikegaya, Saito & Abe, 1996) are densely distributed in these structures.

Classical fear conditioning models have produced a substantial body of evidence to this effect, implicating the activation of GRs and β-adrenoreceptors in the BLA in the consolidation of fear memory (Roozendaal, Okuda, Vanderzee & McGaugh, 2006). Present results suggest that predator stress employs a similar mechanism. Furthermore, predator stress induces LTP-like changes in communication between the amygdala and hippocampus (Adamec, Blundell & Burton, 2005b), providing further support for the role of limbic circuitry in the consolidation of stress effects on behavior.

5. Conclusions

Present findings suggest that β-adrenoreceptors mediate the consolidation of lasting effects of predator stress in the brain. These findings have parallels in clinical models, where symptom severity is reduced following propranolol administration several hours after stress (Pitman, Saunders, Zusman, Healey, Cheema, Lasko et al, 2002; Vaiva, Ducrocq, Jezequel, Averland, Lestavel, Brunet et al, 2003). In contrast, benzodiazepine treatment was without effect in our preclinical model, consistent with previous work in human patients (Gelpin, Bonne, Peri, Brandes & Shalev, 1996). Together, these data pharmacologically validate predator stress as a viable model of PTSD.

Glucocorticoid hormones also act to consolidate stress effects; most likely through interaction with β-adrenoreceptor occupancy. Sensitization of defensive behavior following predator stress has mechanisms in common with fear memory facilitation for an aversive learning task (Roozendaal, Okuda, de Quervain & McGaugh, 2006), as both involve activation of hippocampus and BLA (Adamec, Blundell & Burton, 2005b; Roozendaal, 2003; Roozendaal, Nguyen, Power & McGaugh, 1999). MRs also modulate the effects of predator stress, but differ from GRs in that they lack a role in the emotional facilitation of memory (Roozendaal, 2003).

As it is, activation of GRs, MRs, and β-adrenoreceptors appear to mediate many of the behavioral changes observed following stress. Different behaviors are most likely influenced by differential activation of the various receptor types in multiple brain structures. As such, the role of these receptors in limbic circuitry following predator stress warrants further investigation. Finally, as a parting caveat, blockers (with the

exception of propranolol) were administered in single doses, and the effects of dose on the behavioral syndrome following stress remain to be further explored.

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Figure Captions

Figure 1: Mean + standard error (SEM) or medians (MD, non-parametric analyses) for measures of anxiety-like behavior in all tests but startle following propranolol treatment are plotted. Propranolol was tested in 2 doses, and before and after stress. For each plot, means or medians marked with different letters represent group differences, and those marked with the same letter did not differ. Medians marked with two letters do not differ from medians marked with either of these letters alone.

Figure 2: Mean + SEM or medians (MD) for measures of anxiety-like behavior in all tests but startle following blockade of GR, MR, and combined (GR + β-adrenoreceptor) are plotted. Data from combined predator stressed, combined handled control, and propranolol (5 mg/kg) post-stress are repeated from Figure 1. Mean and median contrast results are labeled as in Figure 1.

Figure 3: Mean + SEM or medians for measures of anxiety-like behavior in all tests but startle following chlordiazepoxide treatment are plotted here. Data from combined predator stressed and combined handled control are repeated from Figure 1.

Figure 4: Effect of stress + vehicle and chlordiazepoxide injection following predator stress on median peak startle amplitude in study 1 appears in the top panel of the figure. The lower panel plots median peak startle amplitude following GR block, and β-adrenoreceptor block before and after stress. In both panels, medians marked with the same letter do not differ, but differ from medians marked differently.

Figure 5: Median peak startle amplitude following treatment with propranolol (10 mg/kg), RU486 + propranolol (5 mg/kg) in combination, and spironolactone are presented. Combined predator stressed and combined handled controls from study 2 are plotted as controls. As before, medians marked with the same letter do not differ, but differ from medians marked differently.

Figure 6: The top panel displays a sample fit for the exponential decline of startle amplitude over trials. The middle panel plots the results of β-adrenoreceptor block both pre and post-stress on τ. The effect of GR block and benzodiazepine receptor antagonism is also presented. τ + SEM values from study 1 handled controls, predator stressed only, and combined stress + vehicle (pre- and post-stress) also appear in the plot. The lower panel displays the impact of β-adrenoreceptor block either alone or in combination with GR block on the stress-induced delay of habituation of startle. The effect of MR block is also presented. τ + SEM values from study 2 combined handled controls and combined predator stressed groups are plotted as well. Again, means marked with the same letter do not differ, but differ from means marked differently. Means marked with two letters fall in between means marked with either letter alone.

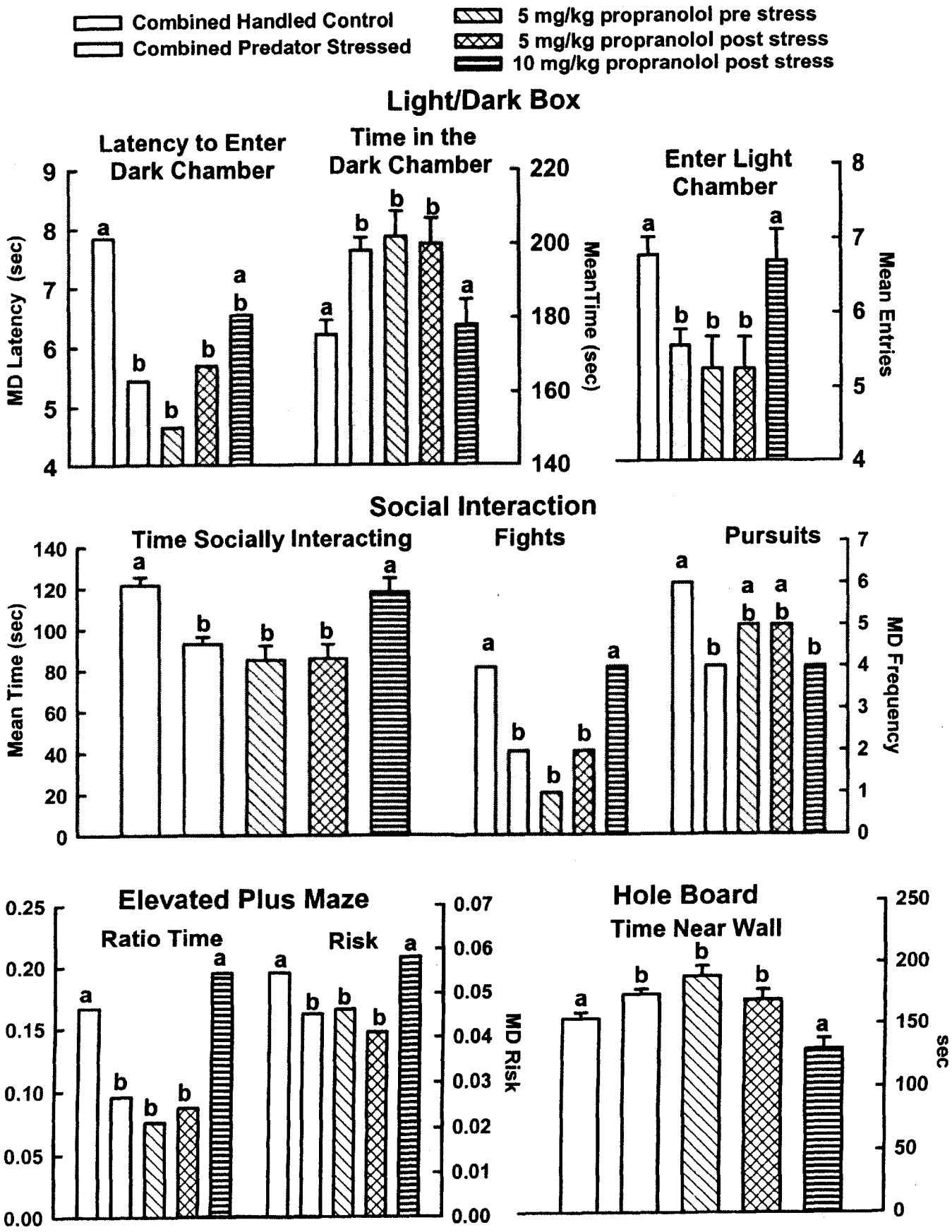

Combined Handled Control
Combined Predator Stressed
5 mg/kg propranolol pre stress
5 mg/kg propranolol post stress
10 mg/kg propranolol post stress
Light/Dark Box
Latency to Enter Dark Chamber
Time in the Dark Chamber
Enter Light Chamber
MD Latency (sec)
MeanTime (sec)
Mean Entries
Social Interaction
Time Socially Interacting
Fights
Pursuits
Mean Time (sec)
MD Frequency
Elevated Plus Maze
Ratio Time
Risk
MD Ratio Time
MD Risk
Hole Board
Time Near Wall
sec

Figure 1

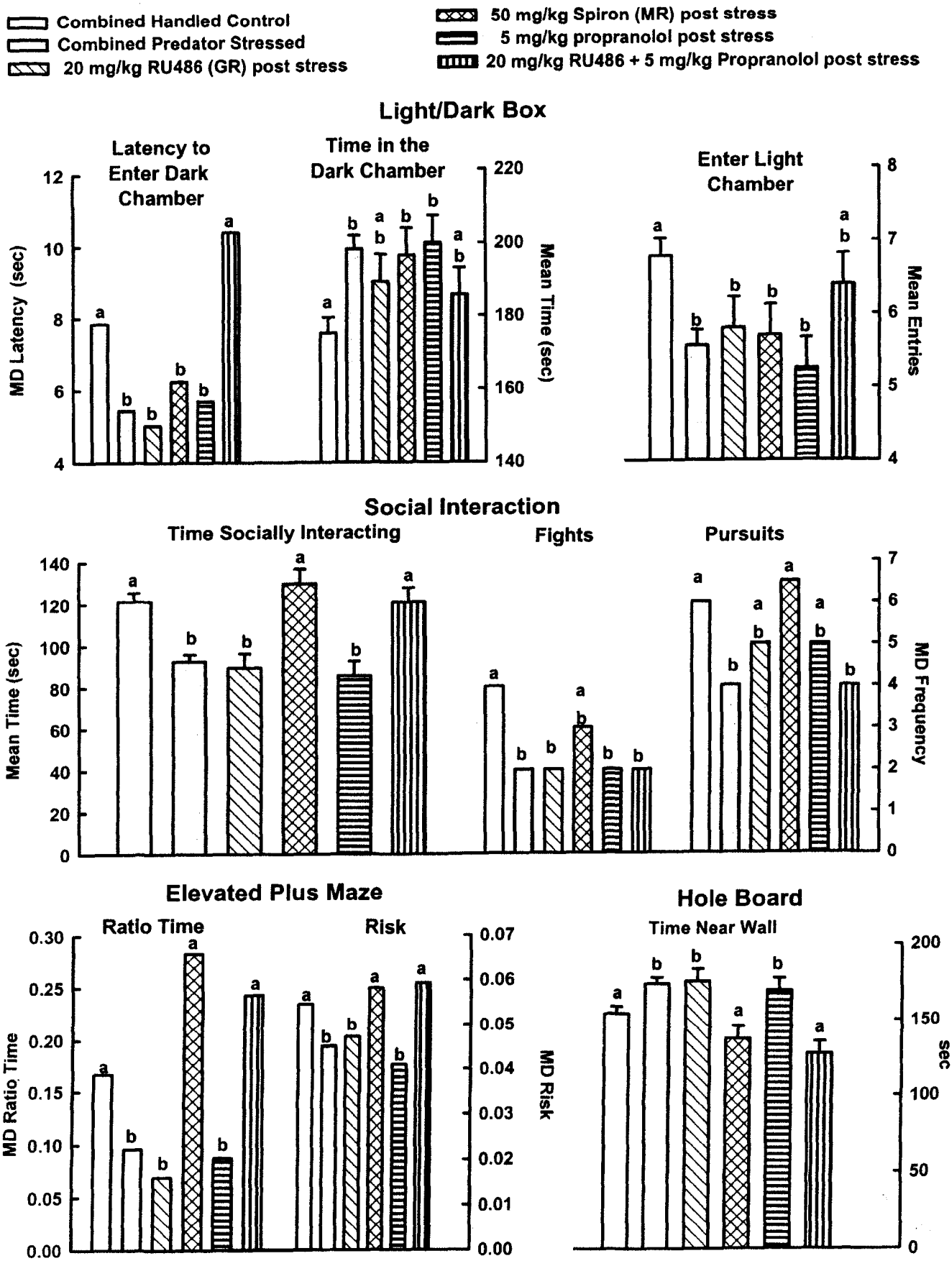

Combined Handled Control
Combined Predator Stressed
20 mg/kg RU486 (GR) post stress
50 mg/kg Spiron (MR) post stress
5 mg/kg propranolol post stress
20 mg/kg RU486 + 5 mg/kg Propranolol post stress
Light/Dark Box
Latency to Enter Dark Chamber
MD Latency (sec)
Time in the Dark Chamber
Mean Time (sec)
Enter Light Chamber
Mean Entries
Social Interaction
Time Socially Interacting
Mean Time (sec)
Fights
Pursuits
MD Frequency
Elevated Plus Maze
Ratio Time
MD Ratio Time
Risk
MD Risk
Hole Board
Time Near Wall
sec

Figure 2

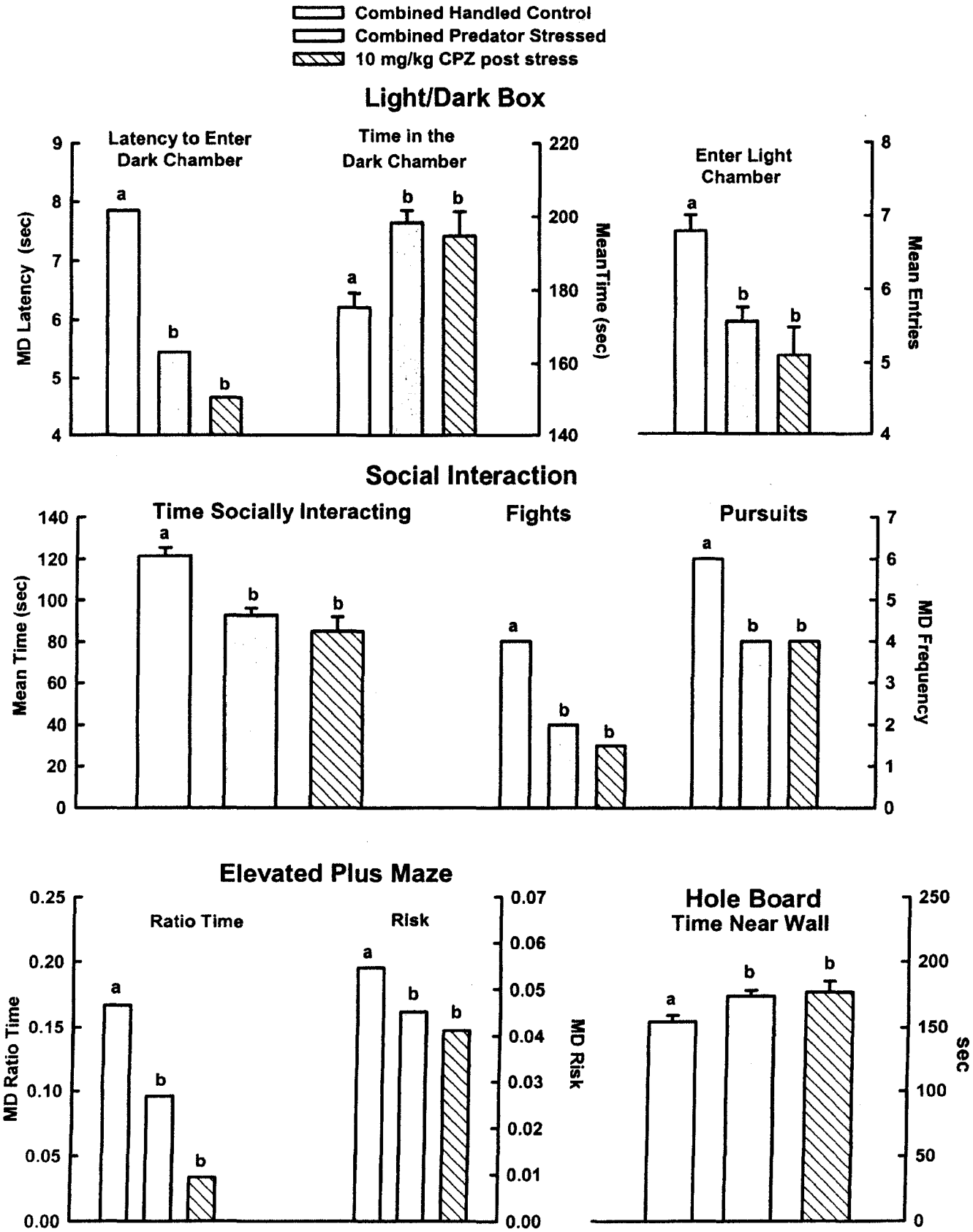

Combined Handled Control
Combined Predator Stressed
10 mg/kg CPZ post stress
Light/Dark Box
Latency to Enter Dark Chamber
Time in the Dark Chamber
Enter Light Chamber
MD Latency (sec)
MeanTime (sec)
Mean Entries
Social Interaction
Time Socially Interacting
Fights
Pursuits
Mean Time (sec)
MD Frequency
Elevated Plus Maze
Ratio Time
Risk
MD Ratio Time
MD Risk
Hole Board
Time Near Wall
sec

Figure 3

Startle in Light and Dark

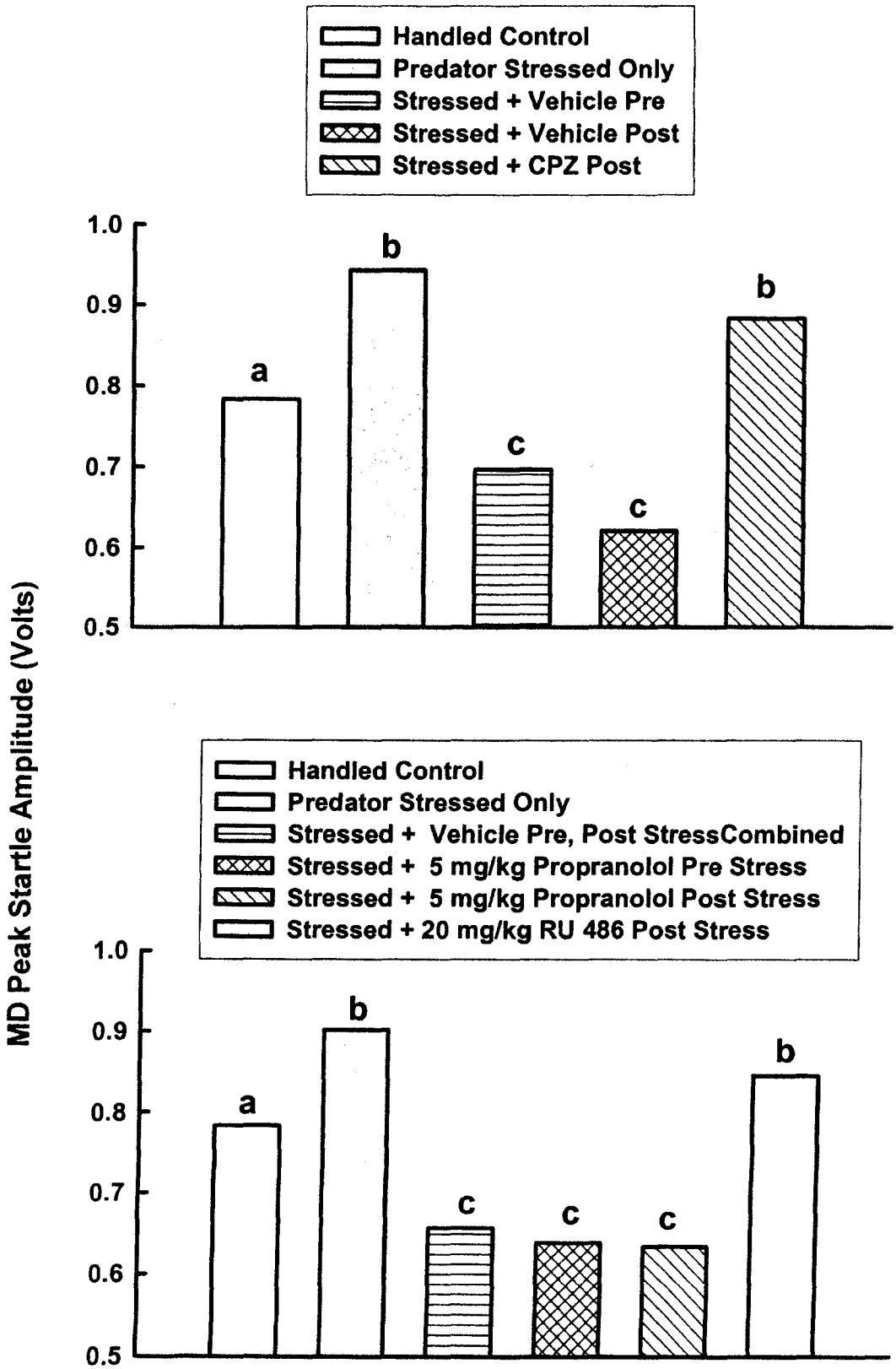


Figure 4

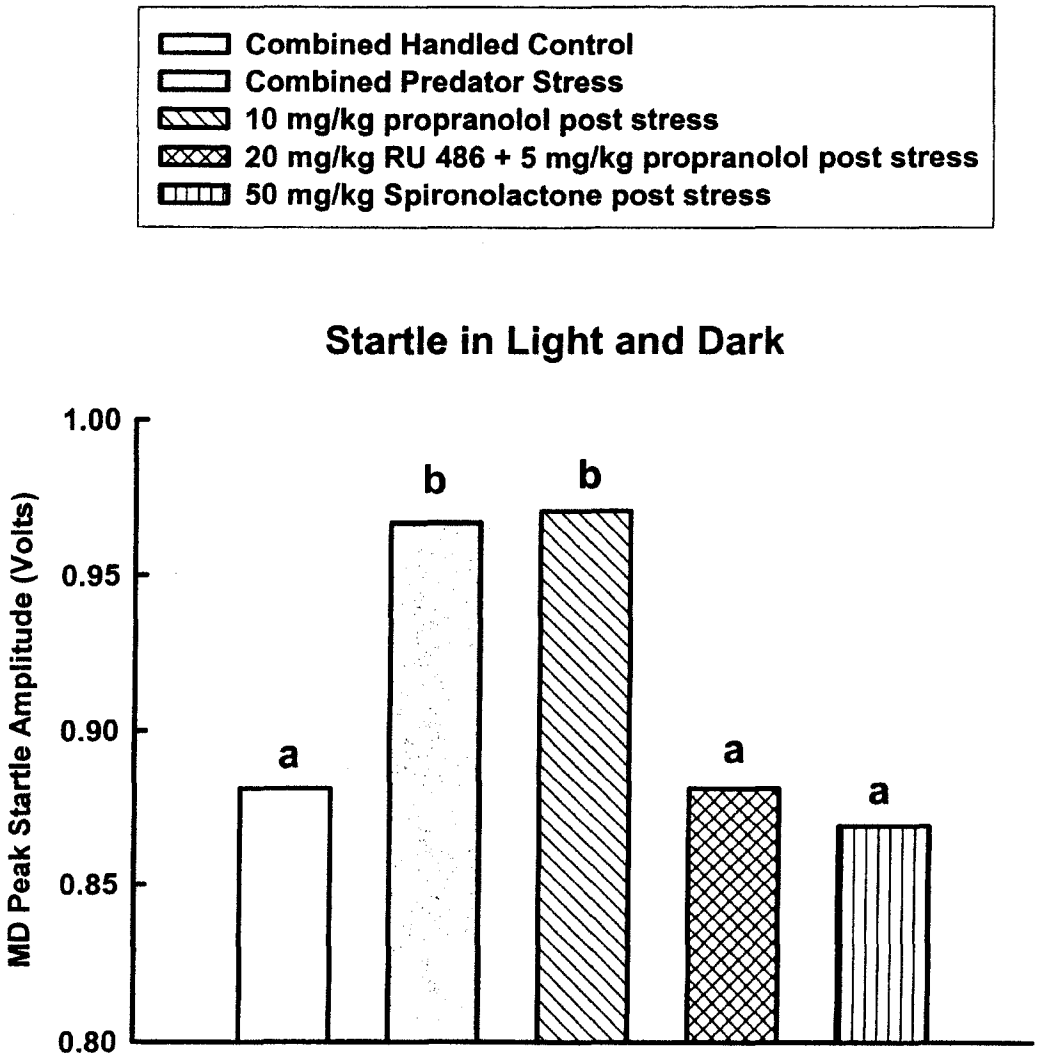
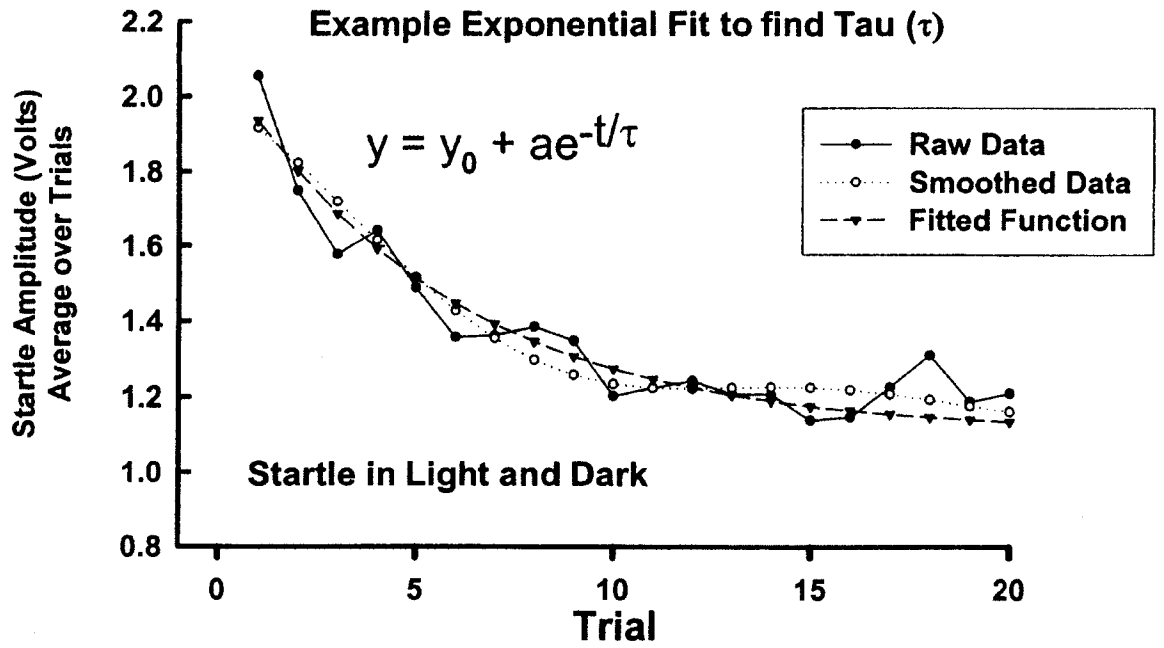
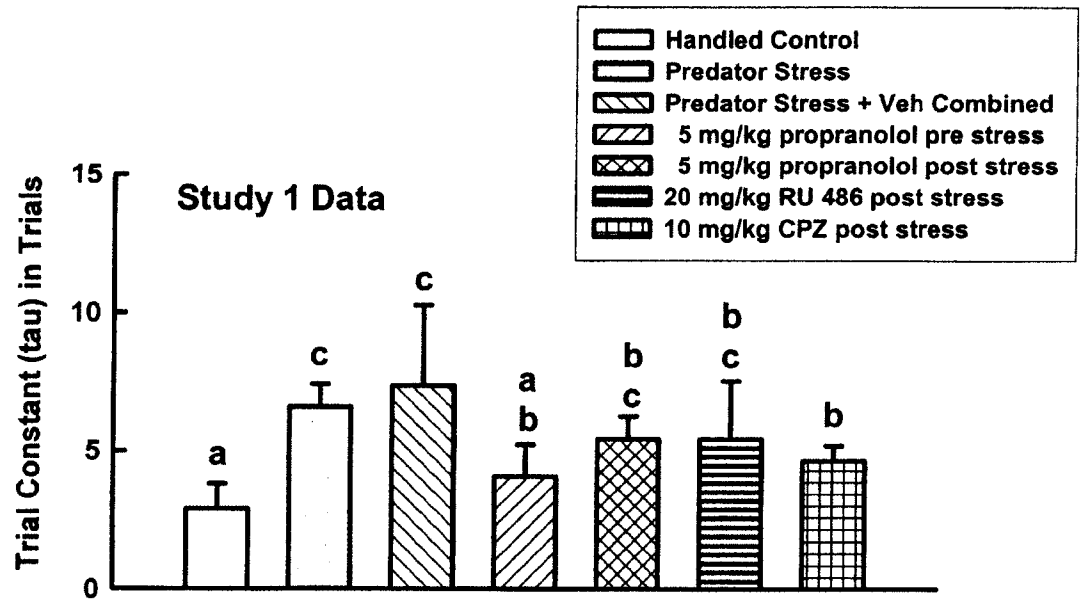

Combined Handled Control
Combined Predator Stress
10 mg/kg propranolol post stress
20 mg/kg RU 486 + 5 mg/kg propranolol post stress
50 mg/kg Spironolactone post stress
Startle in Light and Dark
MD Peak Startle Amplitude (Volts)
1.00
0.95
0.90
0.85
0.80
a
b
b
a
a

Figure 5


Example Exponential Fit to find Tau (τ)
y = y0 + ae-t/τ
Raw Data
Smoothed Data
Fitted Function
Startle Amplitude (Volts)
Average over Trials
Startle in Light and Dark
Trial
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
5
10
15
20


Handled Control
Predator Stress
Predator Stress + Veh Combined
5 mg/kg propranolol pre stress
5 mg/kg propranolol post stress
20 mg/kg RU 486 post stress
10 mg/kg CPZ post stress
Study 1 Data
Trial Constant (tau) in Trials
15
10
5
0
a
c
c
a
b
b
c
b
c
b

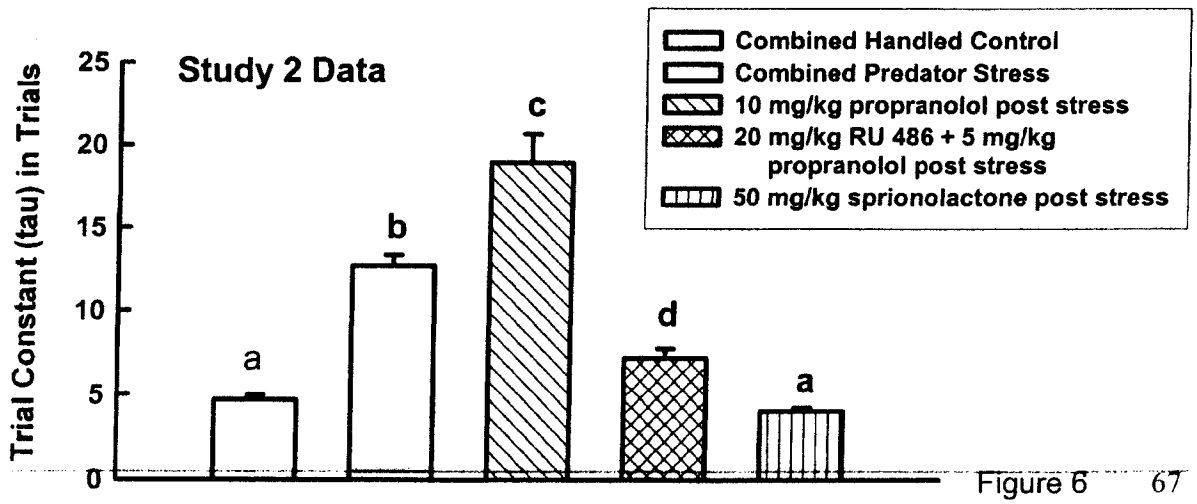

Combined Handled Control
Combined Predator Stress
10 mg/kg propranolol post stress
20 mg/kg RU 486 + 5 mg/kg
propranolol post stress
50 mg/kg sprionolactone post stress
Study 2 Data
Trial Constant (tau) in Trials
25
20
15
10
5
0
a
b
c
d
a

Figure 6