

THE ROLE OF THE READ THROUGH VARIANT OF
ACETYLCHOLINESTERASE IN ANXIogenic EFFECTS
OF PREDATOR STRESS IN MICE

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The Role of the Read Through Variant of Acetylcholinesterase in Anxiogenic Effects of
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by

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Abstract

The goal of this study was to examine the role of the read-through variant of acetylcholinesterase (AChE-R) in the changes in affective behaviour using the predator stress model of PTSD. This read through variant has been shown to exist at higher levels in the brain following stress (Pick, Flores-Flores, & Soreq, 2004, Meshorer et al., 2002).

The role of acetylcholinesterase in predator stress was examined in mice using a novel drug EN101, a systematically administered central acting antisense mRNA for AChE-R. Research by Pollak et al. (2005) demonstrated that cholinergic enhancement using EN101 produces central and peripheral anti-inflammatory effects. EN101 acts to disrupt the stress precipitated induction of the transcription of the read-through variant of AChE by selectively targeting the mRNA sequence for AChE-R. It is AChE-R in limbic cholinergic circuitry that contributes to anxiogenic effects of traumatic stress (Talma et al., 2003). We administered multiple injections of the drug to the same animals at specific time points prior to and after a predator stress exposure in male C57 mice. This was done to ascertain whether the specific action of EN101 on AChE-R expression had any effect on stress induced lasting changes in multiple tests of murine affective behaviour.

Predator stress caused a significant increase in startle amplitude, which EN101 blocked. This effect was specific to EN101, as the control inverse drug INVEN101 was without effect on stress effects on startle amplitude. INVEN101 is the inverse of the EN101 drug

consisting of the same mRNA base pairs only in a different order than EN101. This evidence suggests that EN101 is acting to lower the levels of the read-through variant of acetylcholinesterase in brain regions responsible for startle amplitude (hyperarousal) in rodents. Neither drug affected the impact of predator stress on behaviour in the plus maze, and both drugs partially reduced stress suppression of time active in the hole board. In the light dark box test INVEN101 appeared to exhibit a weak effect partially inhibiting the effects of predator stress on light dark box behaviour. This behavioural change would require replication in order to accept. Together the data reinforce the supposition that multiple neural systems are responsible for the different changes in behaviour produced by predator stress.

This study provides evidence for a role of AChE-R in specific changes in anxiety-like behaviour following stress. Further research is necessary to pinpoint the exact time window for administration of the drug in order to prevent or inhibit changes in affective behaviour following predator stress. Work is also needed to determine whether other systemic effects of the drug might occur.

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The Role of the Read Through Variant of Acetylcholinesterase in Anxiogenic Effects of Predator Stress in Mice

Post-traumatic stress disorder (PTSD) is an incapacitating anxiety disorder resulting from exposure to a traumatic life experience. PTSD is characterized by a number of modifications in stress-related neurotransmitter, neurohormonal, and immune system functions. In the biological model of PTSD, exposure to traumatic stress is followed by a failed neuroendocrine adaptive response to the traumatic event, which results in changes in affect (Yehuda et al., 2001). Unfortunately, despite a wealth of effort there is still uncertainty surrounding the neurological basis of the disorder. How little we actually know about the prevention and treatment of PTSD following a traumatic event was recently illustrated by research which found that critical incident stress debriefing (CISD), the major intervention following 9/11, was relatively ineffective and may have actually been more harmful than helpful (Pomerantz, 2006). Current intensification of geo-political conflict and the heightened risk of terrorist attacks in the global community have increased the threat of traumatic events or stressors. Therefore there is a growing need for research into treatments for PTSD.

According to the American Psychiatric Association's (APA) Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), in the aftermath of trauma, victims may exhibit symptoms that include re-experience of the traumatic event, poor emotional coping or avoidance, and an exaggerated arousal response or hypervigilance. The DSM-IV outlines six specific criteria of which at least two must be present for a diagnosis of PTSD. These criteria include exposure to a traumatic event where the subject exhibited

intense fear, persistent reexperience of the traumatic event, persistent avoidance of the associated stimuli, symptoms of increased arousal such as hypervigilance and insomnia, disturbances causing clinically significant distress such as social impairment, and finally a duration of symptoms of at least one month. The American Psychiatric Association further differentiates the diagnosis of PTSD based on the duration of the symptoms. The disorder is characterized as acute when symptoms persist for less than 3 months. If the duration is 3 months or more, then a diagnosis of chronic PTSD is made (APA DSM-IV).

The average lifetime prevalence rate of PTSD in the general population has been estimated at between 7 and 9 percent (Frans, Rimmö, Åberg, & Fredrikson, 2005, Kessler et al., 1995, Breslau et al, 1995). Moreover, further evidence indicates that up to thirty percent of individuals exposed to an acute traumatic experience develop PTSD, however that likelihood depends on the intensity and kind of traumatic exposure as well as on individual resilience (Pomerantz, 2006). Interestingly, certain people run a higher risk of developing PTSD than others following a traumatic experience. Studies indicate only a proportion of subjects will develop the disorder implicating other factors such as genetics in the etiology of the disorder (Cohen et al. 2003). Women have twice as high a risk as men, and sexual and physical abuse during childhood may sensitize the nervous system, which then overreacts and perseverates when exposed to traumatic events in adulthood (Pomerantz, 2006).

Genetic factors have been implicated recently in the etiology of PTSD. Twin studies have demonstrated that genetic factors play an important role in the vulnerability to develop PTSD (Seedat, Niehaus, & Stein, 2001). While the exact genetic factors that

influence PTSD susceptibility have not been fully identified, Stein et al., (2002) suggest that genetic influences on PTSD are presumably mediated through a causal pathway that includes genes that simultaneously influence personality (i.e. exposure proneness - traits which increase one's likelihood to be exposed to traumatic events) and PTSD symptoms following exposure.

Studies suggest that a genetic predisposition may result in changes in neural systems making people more susceptible to developing the disorder. According to Sternfeld et al., (2000) the cellular and molecular factors that mediate the switch between physiological accommodation and neurological disease likely reflect complex interactions between the genetic background of the individuals and the nature of the stress insult.

Developing effective treatments for PTSD is an important area of research. With the current frequency of traumatic events occurring worldwide and the increasing threat of terrorist activities substantiated by the attacks of 9/11, it is likely that the lifetime prevalence rates of PTSD may climb. In order to treat PTSD effectively the neurological mechanisms that underlie the disorder must be fully understood. However a major challenge for research into the mechanisms of PTSD is identifying the molecular mechanisms linked to changes in affect that underlie the enhanced formation of memory following stress exposure (Nijholt et al., 2004).

There is growing evidence that the neural plasticity underlying PTSD involves integrated actions of neuronal systems such as the cholinergic, noradrenergic and serotonergic neural circuitry involved in emotion and memory and other fear memory

related structures such as the amygdala and hippocampus (Adamec, Walling, & Burton, 2004, Pick et al., 2004, and Morilak et al., 2005). Drugs currently used to treat symptoms of PTSD, such as benzodiazepine agonists and selective serotonin re-uptake inhibitors (SSRI's) such as fluoxetine (Prozac), each act on a different neurotransmitter system (Degroot, A., & Nomikos, G., 2005, Adamec, Creamer, Bartoszyk, & Burton 2004, Adamec, Bartoszyk, & Burton, 2004). This evidence suggests that multiple neural systems may be involved in the precipitation of the various changes in behavioural affect observed following a traumatic event (Adamec, Blundell, & Burton, 2006, McIntyre, Power, Roozendaal, & McGaugh, 2003). The development of animal models will be critical for clarifying the cascade of events that precipitates the onset of PTSD.

Models of Lasting Impact of Stress on Brain and Behaviour

Despite a broad body of evidence concerning the neurobiological correlates of PTSD, the neuronal mechanisms of PTSD are still poorly understood. This illustrates the importance of animal models of this disorder. Animal models provide an invaluable tool in the study of the neural mechanisms that underlie affective disorders such as anxiety and PTSD and may contribute to the development of new medications to treat psychiatric disorders. Recently, animal model has become a somewhat fashionable term used in animal studies for almost every stress-induced behavioral alteration. Only few cases, however, reflect the human disorder closely enough to be truly called an animal model of PTSD (Seigmund and Wotjak, 2006).

A good animal model of a complex human clinical disorder must strive to parallel the clinical conditions as closely as possible (Cohen et al., 2004). Systematic research

requires valid animal modeling with clearly defined criteria that can be measured and quantified. Such models permit researchers to explore aspects of the disorder, which would be impossible in human studies for ethical or practical reasons. In addition, animal studies permit the researcher a level of control that is unattainable in human studies.

There are a number of different animal models of lasting effects of stress on affect relevant to PTSD used to further scientific understanding of the disorder as well as to aid in the development of new treatments (Rau, DeCola, & Fanselow, 2005). Among these animal models are: underwater trauma, exposure of a rodent to predator stress, inescapable electric shock and fear conditioning. An important criterion for a valid animal model of PTSD is that it produces long-lasting quantifiable changes in affective behaviour.

Studies of classical fear conditioning require the recognition of a conditioned stimulus (CS) and the association of the CS with an aversive stimulus (UCS). Such studies have found that amygdala neural plasticity underlies both the acquisition and extinction of fear responses to simple and complex sensory (contextual) stimuli (Adamec et al., 2006, Blair et al., 2001). Moreover, Rau et al., (2005) have shown that pre-exposure to a stressor of repeated foot-shock enhances conditional fear responding to a single context-shock pairing, mimicking the clinical finding that stress history intensifies PTSD symptoms following a trauma. The conditioning model shows promise in facilitating the study of neurobiological mechanisms underlying memory-based symptoms such as re-experiencing the traumatic event. Moreover, novel post stressor interventions to alleviate subsequent PTSD symptoms in humans have arisen from the

study of the role of catecholamines in fear conditioning (McIntyre et al., 2003, Southwick, Page, & Morgan, 1999, Kobayashi, K., 2001).

In rats, intraamygdala infusion of the β -adrenoceptor antagonist propranolol blocks the glucocorticoid facilitation of fear memory consolidation when administered shortly after contextual fear conditioning (McIntyre et al., 2003). Propranolol acts by binding to peripheral and central β -adrenergic receptors and readily crosses the blood brain barrier (Vaiva et al., 2003). This antagonist also blocks the memory-modulating effects of other neurotransmitter systems, indicating that their effects on memory consolidation are also mediated through noradrenergic activation within the amygdala (McGaugh. et al., 2002). Further evidence indicates that the administration of propranolol attenuates the enhanced long-term memory induced by emotionally arousing information without affecting memory for neutral information, which suggests that over activation of this modulatory system may contribute to the development of PTSD (McIntyre et al., 2003). Recent clinical research by Pitman et al. (2002) has demonstrated that propranolol administered within 6 hours of the traumatic stress and continuing over 10 days was superior to a placebo for reducing PTSD symptoms 1 month post-trauma. Vaiva et al. (2003) later replicated these findings in a similar study.

The other animal models such as underwater trauma and predator stress focus on exposing animals to a traumatic event such as a predator exposure or being forced underwater to produce lasting changes in affect analogous to the fear component of human anxiety. It has been well documented that such life-threatening inescapable stresses lead to lasting change in affective functioning (Adamec, & Shallow, 1993, Cohen

et al., 1996, Richter-Levin, 1998, and Van der Kolk, Greenberg, Boyd, & Krystal, 1985). Moreover, propranolol administered post predator stress blocks the stress induced lasting increases in rodent anxiety (Adamec, Muir, & Pearcey, 2007). These findings add pharmacological validity to predator stress as a model of PTSD.

There is growing evidence for predator stress as a credible model of certain aspects of PTSD. First, predator stress possesses ecological validity as a natural life threatening stressor to rodents (Adamec et al., 2006, Cohen et al., 2004, Belzung, Hage, Moindrot, & Griebel, 2001). Second, research has consistently demonstrated that predator stress induces long-term increases in rodent anxiety-like behaviour, lasting over a month following a brief exposure to a cat (Adamec & Shallow, 1993, Adamec et al., 2005, Cohen et al., 2003, and Hage and Belzung, 2002). It has been suggested that viewed as ratio of lifespan, the duration of predator stress effects on affect in animals models the duration of some symptoms of chronic PTSD in humans (Adamec, 1997, Adamec et al., 2006).

Among the lasting changes in affect following predator stress is an increase in acoustic startle amplitude, which is very similar to the hypervigilance or hyperarousal symptoms seen in PTSD (Adamec, 1997). Aside from being a good model of generalized sensitization/hyperarousal, there is evidence to suggest the predator stress model may also model the avoidance of trauma reminders seen in humans suffering from PTSD, that is the avoidance of open spaces in the elevated plus maze may be reminiscent (i.e. trauma reminder) of the open space of the room in which the cat exposure took place (Adamec et

al., 2006). The predator stress model has been demonstrated in both mice and rats (Adamec & Walling, 2004, Belzung et al, 2001).

Brain Mechanisms: Stress Effects on Affective Behaviour

The amygdala appears to modulate the consolidation of long-term explicit memories of emotionally arousing experiences by influencing other brain regions shown to be involved in changes in affect following stress, such as the hippocampus, caudate nucleus, nucleus basalis, and cortex (Gulpinar & Yegen, 2004). McGaugh and Roozendaal (2002) illustrated how neuroendocrine response to stressors modulates amygdala circuitry that is involved in associative fear conditioning. This may be part of the process by which stressors produce the indelible fear memories associated with PTSD (Adamec et al., 2006).

Positron Emission Tomography (PET) studies of Vietnam War veterans have revealed that activation of the right amygdala occurred following stimuli associated with wartime trauma suggesting that the right amygdala may be particularly important in the mediation of PTSD symptoms (Shin et al., 1997). In an analogous fashion, evidence is accumulating indicating neuroplastic change in right hemispheric brain regions in changes in affective behaviour produced by predator stress. Neuroplasticity in the afferents to the right amygdala from the hippocampus and right amygdala efferents to periaqueductal gray (PAG) have been implicated in preclinical studies using the predator stress model of PTSD (Adamec et al., 2005).

Neuronal plasticity is one of the fundamental processes that occurs following a stressful event in an attempt to make the appropriate adaptive response in similar

situations in the future (Gulpinar & Yegen, 2004). However, following stress neuronal plasticity may occur in such a manner that it causes changes in neural substrates which are no longer adaptive, resulting in psychological disorders such as PTSD. Such neural plasticity in brain regions including the amygdala and the hippocampus are thought to mediate affective psychopathology (Layton and Krikorian, 2002, Adamec et al., 2006).

Stress precipitated neural plasticity may occur as a result of a chemical cascade involving phosphorylated cyclic AMP response element binding protein (pCREB), N-methyl-D-aspartate (NMDA) receptors and long-term potentiation (LTP) (Rau et al., 2005). Evidence suggests that acute stress (predator stress) produces NMDA dependent LTP of amygdala afferent and efferent transmission which is highly predictive of anxiogenic effects of stress (Adamec, Blundell & Burton, 2003). Blocking NMDA receptors before but not after predator stress has also been shown to prevent lasting increases in anxiety-like behaviour in rodents following stress, likely by precluding the chemical cascades involved in LTP (Blundell & Adamec., 2006).

As mentioned above the PAG is an area of the brain that has been linked to the neural plasticity underlying anxiety and PTSD. Predator stress appears to increase the degree of phosphorylated cyclic AMP response element binding protein (pCREB) expression in the PAG that is associated with stress induced long lasting LTP of central amygdala to lateral column of the PAG (ACE-PAG) transmission (Adamec et al., 2003). Furthermore, stress induced LTP of ACE-PAG transmission appears to mediate some of the changes in rodent affect following stress. In support of this view, the same aspects of the stressor experience and reaction to it, which are predictive of the degree of pCREB

expression, are also highly predictive of the degree of potentiation of ACE-PAG transmission. Moreover, covariance analysis suggests that ACE-PAG potentiation mediates some but not all of the changes in affective behaviour produced by predator stress since removing behavioural variance predicted by ACE-PAG LTP eliminates stress effects on behaviour in the plus maze and in acoustic startle but not in other behavioural measures (social interaction and light dark box) (Adamec et al., 2003).

According to Adamec et al., (2006), pCREB in the right PAG may be part of the chemical cascade which leads to long lasting neural plasticity (LTP) in the PAG. The PAG has also been shown to contain cholecystokinin (CCK) immunoreactive fibers and CCK (2) receptors which have been previously implicated in anxiety disorders (Bertoglio & Zangrossi, 2005). Moreover systemic block of CCK (2) receptors post predator stress blocks stress induced anxiogenic effects (Adamec, Burton, Shallow, & Budgell, 1999).

The cholinergic system has more recently been linked to PTSD (Benson, 2004, Gulpinar et al., 2004, Pick et al., 2004). Evidence implicates the hippocampal cholinergic system as a site where anxiety and memory converge (Degroot and Nomikos, 2005). The hippocampus is a critical component of the neuroanatomical stress circuit as well as many other vital brain functions (Nijholt et al., 2004). Cholinergic neural circuitry connecting the hippocampus and amygdala in parallel with other structures is thought to mediate some of the symptoms exhibited by people suffering from PTSD (Sklan et al., 2004, Degroot and Nomikos, 2005). Fluctuations of acetylcholine (ACh) efflux in the hippocampus are associated with the modulation of emotionality and cognition (Egawa et al. 2002, Degroot and Treit, 2002). Degroot and Nomikos (2005) suggest that increases

in hippocampal ACh are related to the emotional impact of an event and the memory of that event. Moreover nicotinic and muscarinic ACh receptors in the hippocampus are required for the expression of LTP (Gulpinar and Yegen, 2004). Cholinergic receptor activation enhances the responsiveness of NMDA glutamate receptors and facilitates the induction of LTP (Gulpinar and Yegen, 2004).

Cholinergic processes that modulate memory in the hippocampus appear to be mediated selectively by the basolateral complex of nuclei in the amygdala (Power, Bazarjanova, & McGaugh, 2003). Muscarinic cholinergic activation within the amygdala also appears to be critical for enabling emotional memory-modulatory influences in other areas of the brain such as the hippocampus, striatum, nucleus basalis and cortex (McGaugh et al., 2002). Taken together these data implicate cholinergic activation in the amygdala as an essential step in the cholinergic neural cascade underlying stress.

Current Investigation of EN101 and the Cholinergic System

ACh is the neurotransmitter of the basal forebrain cholinergic neurons that are associated with cognitive processes involved in memory and emotion. These neurons innervate limbic structures purportedly involved in PTSD including the hippocampus, anterior cingulate cortex and the amygdala (Gulpinar & Yegen, 2004). To date, research has not examined the role of ACh function in the predator stress model of PTSD, although there is evidence linking ACh function to fear learning, neuroplasticity and stress. Moreover, the cholinergic system is implicated in normal and pathological regulation of emotion (Benson, 2004).

Acetylcholinesterase (AChE) is the enzyme responsible for catalyzing the hydrolysis of ACh in the brain (Fu, Zhang, & Sun, 2005). AChE has been implicated in the neurophysiology of stress effects on affect (Kaufer, Friedman, Seidman, & Soreq, 1998, Nijholt et al., 2004, Meshorer et al., 2002). Stress induces a shift from the neuronal primary splice pattern yielding AChE-S to the hydrophilic AChE-R (Pick et al., 2004). Each of these AChE variants is specialized: AChE-S for the hydrolysis of acetylcholine at the synapse, and AChE-R for non-synaptic hydrolysis and morphogenesis (Brenner et al., 2003). Under usual neural conditions, the accumulation of excess neuronal AChE-R in response to stress assists in the removal of additional ACh molecules to help restore cholinergic homeostasis. However, the lack of a C-terminal cysteine in the molecular structure of the readthrough splice variant (AChE-R) prevents it from adhering to the synaptic membrane forcing it remain intracellular and compete with its protein homologue, neuroligin, in excitatory synapses on interaction with β -neurexin. As a result in the long term, excess neuronal AChE-R may become detrimental to brain functioning (Pick et al., 2004).

AChE-R, normally the least abundant of the alternative splice variants, is thought to restore normal cholinergic activity following a stress response (Pick et al., 2004). Stress increases the transcription of AChE-R, which in turn facilitates neuroplasticity in limbic structures resulting in long lasting changes in neural systems (Meshorer et al., 2002, Nijholt et al., 2004, Kaufer et al., 1998, Cohen et al., 2002, Sternfeld et al., 2000). Thus, modulated cholinergic gene expression may play a crucial role in short-term suppression of brain activity following a traumatic experience but could have potentially

damaging long-term implications (Kaufer et al., 1998). It has been proposed that following certain traumatic or stressful events, AChE-R increases to a level which is no longer adaptive and results in physiological impairments linked to the changes in affect seen as anxiety and PTSD (Cohen et al., 2002, Sembulingam, Sembulingam, & Namasivayam, 2003, Meshorer et al., 2002, Pick et al., 2004, Grisaru et al., 2000).

Other molecules may mediate the upregulation of AChE-R and its subsequent action in the brain following stress. For example, glucocorticoids play a role in the regulation of the cholinergic system following stress. Cortisol release in humans following stress upregulates AChE in addition to enhancing AChE gene expression and possibly elevates AChE-R levels abnormally (Cohen et al., 2002, Battaglia & Ogliari, 2005). In rodents corticosterone induces the accumulation of ARP (Acetylcholinesterase Readthrough Peptide), the 26 amino acid C-terminal domain of the read-through variant of acetylcholinesterase (AChE-R) (Grisaru et al., 2001).

Birikh, Sklan, Shoham, & Soreq (2003) discovered that the readthrough variant of AChE forms tight, coimmunoprecipitable triple complexes with RACK1 and PKC β II, and facilitates stress induced PKC β II accumulation, which is associated with prolonged conflict behaviour patterns. This interaction is also thought to increase read-through acetylcholinesterase's enzymatic activity and enlarge its density in hippocampal neurons. RACK1 is a relatively recently discovered intracellular scaffold protein that interacts with the C-terminal of AChE-R, aiding in the translocation of the enzyme to the cell nucleus in the brain. PKC β II is a protein kinase known to be involved in fear conditioning (Nijholt et al., 2004, Sklan, Podoly, & Soreq, 2006, Birikh et al., 2003).

AChE-R may exert its effect on neural processes following stress both via its effect on acetylcholine transmission as well as through its interaction with RACK1 and PKC β II. The overproduction of AChE-R facilitates synaptic plasticity through a process involving these two molecules, potentially enhancing contextual fear memory (Nijholt et al., 2004). The involvement of AChE-R in contextual fear memory may be relevant to neural changes underlying anxiogenic effects of predator stress.

Studies of both fear conditioning and predator stress have suggested a role for LTP in the hippocampal ventral angular bundle efferent to the basolateral amygdala (VAB-BLA) in increased fearfulness (Maren & Fanselow, 1995, Adamec, 2001; Adamec et al, 2003; 2005). Moreover, electrolytic lesions placed in regions of the hippocampus that project to the BLA or excitotoxic lesions placed in the BLA eliminated contextual fear conditioning demonstrating the critical role of both structures in the neural plasticity underlying this form of fear learning (Maren & Fanselow., 1995). Similarly unprotected exposure of rats to cats produces LTP in right VAB-BLA transmission and LTD like changes in the left (Adamec et al., 2001). There may be a link between plasticity in hippocampo-amygdala transmission and AChE-R given evidence of stress induced overexpression of the AChE-R protein in neurons notably in the dentate gyrus and BLA (Sternfeld et al., 2000).

The present study is an initial attempt to determine if altered expression of the AChE-R variant is involved in the lasting anxiogenic effects of predator stress. To do this, the effects of a novel drug, EN101, in blocking lasting changes in rodent affect produced by a predator stress (unprotected exposure to a cat) were studied. Recent

findings suggest predator stress induced plasticity in neural (limbic system) circuitry, which is implicated in fear learning, underlies some of the anxiogenic effects observed following a predator stress event (Adamec et al., 2005). EN101 may act to disrupt stress precipitated chemical cascades involving AChE-R in the limbic cholinergic system which may contribute to the anxiogenic effects of stress (Talma et al., 2003).

EN101, developed by Dr. Soreq in Jerusalem, is a systematically administered, central acting antisense oligonucleotide selectively targeting mRNA for AChE-R in the brain. Administration of EN101 selectively lowers the level of the read through splice variant of acetylcholinesterase (AChE-R) in stress responsive brain regions including the hippocampus and amygdala (Brenner et al., 2003, Pollak et al., 2005). It has recently been found that AChE-R mRNA, having a long 3' untranslated domain, is significantly more sensitive to antisense interference than the synaptic transcript, explaining the selective action of this drug (Cohen et al., 2002). INVEN101 is the inverse of EN101 and is used as a control in this study. INVEN101 contains the same mRNA nucleotides but in a different sequence and has been shown to have no effect on AChE-R transcription and no effects on other systems have been reported (Nijholt et al., 2004).

The current study attempts to determine whether blockage of AChE-R transcription with EN101, administered at multiple intervals (24 hours prior to predator stress, 10 minutes post stress, as well as 24 and 48 hours post stress), will reduce the anxiogenic effect of predator stress in mice 7 days post stress. Multiple injections were administered as this study is an initial look aimed at capturing both initiation and

consolidation. In addition research by Nijholt et al., (2004) shows that AChE-R protein returns to baseline within 24 hours.

Methods

Subjects

One hundred and fifty male C57BL/6J mice served as test subjects. Upon arrival the mice were six to eight weeks of age and weighed between 20 and 30g. Mice were housed individually in polycarbonate cages measuring 20cm x 15cm x 10cm with continuous access to food and water. Mice were placed on a two-week reverse light cycle adaptation period that consisted of the lights turned off at seven am, and lights on at seven pm. All mice were handled once per day for three days prior to the initiation of testing procedures. Handling involved picking up the mice with a gloved hand one at a time and holding them for one minute on the handler's forearm with minimal pressure, then placing them back in their cages.

Groups

The mice were randomly assigned to one of six different groups consisting of 25 animals per group. The six groups were Handled Control (H), Handled Vehicle (HV), Predator Stressed (EXP), Predator Stressed Vehicle (EXPV), Predator Stressed EN101 (EN101), and Predator Stressed INVEN101 (INVEN101). Prior to the first day of pre-startle testing in week two, all animals were treated the same. During week three each group was exposed to a specific treatment regimen.

Treatment schedule. During week three, the mice were exposed to their treatment schedule. Two mice from each group for a total of twelve mice were tested per week for the duration of the study. During the final week of the study one mouse from each group were tested. The first day of manipulation occurred on Monday at which point all animals in the drug or vehicle groups were given an injection 24 hours prior to treatment. All handling and predator stress cat exposures were then carried out on Tuesday of each week starting at 10:55am and continuing in sixteen-minute intervals. Further injections were administered precisely 10 minutes post treatment, 24 hours post treatment (Wednesday), and 48 hours post treatment (Thursday). Research by Soreq and colleagues suggests that there may be a 24-hour window during which the drug is effective. They demonstrated that EN101 treatment relieved the decremental compound muscle action potential (CMAP) response for a 24-hour period (Brenner et al., 2003). Therefore the handling or predator stress events were carried out 23 hours following the first injection. Injections were carried out post treatment as well to ensure continued post stress drug action. The injection volume of the two drugs used was a standard .28 ml containing a dose of 500 μ g/kg. A .28 ml injection of saline was administered to the handled vehicle and predator stressed vehicle groups. The order in which animals from each group were injected and exposed/handled was randomly set each week to control for time and order effects.

Handled Groups (H, HV). Mice in the handled control and handled vehicle groups did not come into contact with the cats, cat odour, or other mice that had been in contact with the cats and/or their odour. This was to ensure a controlled baseline measure

of mouse behaviour in the upcoming battery of behavioural tests. Mice were handled for one minute on the day of cat exposures of other groups. All mice were handled in the same room as they were housed. Mice in the handled vehicle group also received .28ml injections of saline solution following the testing treatment procedure described above in a different room from other procedures.

Predator Stressed (EXP, EXPV, EN101, INVEN101). The cat exposures took place in a room measuring 160 cm wide by 183 cm long with carpet on the floor marked off into 1-foot squares with autoclave tape. Throughout the study four cats were randomly assigned to the different groups to ensure an equal representation of each cat in all groups to control for differences in cat reaction to the mice.

The predator stress exposures were 10 minutes in duration with the cat being placed in the room approximately 5 minutes before the exposure began. The mice were transported to the exposure room using a small polycarbonate box measuring 10cm x 10cm x 8cm. The polycarbonate enclosure was then positioned at a small trap door entrance to the room, which permitted the subjects being placed in the room without handling.

At the end of the ten-minute exposure mice were removed from the room by gently guiding the mice back into the box using a soft broom. The cat was left in the room until the mice had been returned to their home cages. All mice were examined for wounds following the interaction with the cat and no injuries were apparent. All tests were videotaped for later analysis of behavioural responses of both the mice and cat.

These responses ranged from sniffing, approaching, defensive attacks, escapes, and pursuits.

Behavioural Tests and Measures

A battery of behavioural tests was used to examine anxiety-like behaviour in the mice following treatment. These tests included the acoustic startle response, hole board, plus maze, and the light dark box. Treit, Menard, and Royan, (1993) and Bouwknecht and Paylor (2002) previously demonstrated that these tests are valid measures of anxiety-like behaviour in rodents and predator stress has been shown to lastingly affect behaviour in these tests in mice (Adamec & Walling, 2004). With the exception of startle pre testing, all post treatment behavioural testing took place on the Friday of week three during the hours of 9:00AM and 11:30AM, 9 days after treatment and 7 days after the last injection for the injected mice. All tests were videotaped for later analysis.

Startle. All startle testing was performed in a San Diego Instruments standard startle chamber apparatus. The mice were positioned using a gloved hand into a clear Plexiglas cylindrical enclosure that was then placed inside the startle box. The chamber measured 12.7 cm long and 3.7 cm in diameter. All mice were weighed prior to startle testing.

On Monday, Tuesday, and Friday during the second week that the mice were housed at the research facility, all animals went through pre treatment startle habituation that consisted of ten dark trials. These pre startle tests occurred between the hours of 8:00am and 12:00pm on each of the three days. Mice were first adapted to the startle

chamber for 5 minutes using a background of 50 decibels of white noise. Following this acclimation period mice were exposed to 10 pulses of 50 millisecond bursts of white noise of 105-decibel amplitude rising out of a background of 50 decibels of white noise. There was a 30 second inter trial interval with a 150 millisecond recording window. All startle tests took place in the dark. Assessment of the post treatment startle response occurred on Friday of week three. The same parameters were applied as in the pre stress startle with the exception of an additional ten light trials randomly interspersed with the ten dark trials. For light trials, lights in the startle chamber came on for 2.95 seconds prior to the startle stimulus, and remained on for the duration of the startle stimulus. At three seconds both the startle stimulus and the light were turned off. The light intensity in the chamber at the level of the mouse enclosure was equivalent to 28-foot candles.

Startle Response measures. A computer connected to the startle chamber during the pre and post stress startle testing recorded four measures of startle response. The initial measure recorded was V_{start} defined as the baseline voltage recorded in the first millisecond of the response window prior to initiation of the acoustic startle stimulus. V_{max} was taken as the point of highest voltage or peak response within the 150 millisecond response window. Subtracting the V_{start} value from the V_{max} value for each trial then produced derived peak startle amplitude.

Rate of decline of peak startle amplitude was measured by fitting exponential declining functions on average peak startle amplitude over trials of each group separately (Table Curve, Jandel program). Raw data were smoothed with an FFT function (15% smooth) to improve the fit (Figure 6). The fit provided parameter estimates of the

equation: $y = b + ae^{-T/\tau}$; where y is startle amplitude, b and a are constants, T is trial and τ is the trial constant (tau). Tau is the number of trials it takes for peak startle amplitude to decline to 37% of the maximum and is a measure of rate of startle habituation. Estimates of tau included a standard error which was used to calculate t test values when comparing different estimates of tau.

Hole Board. The hole board apparatus consisted of an open, square, wooden box with black walls and plastic light-coloured flooring. The walls of the hole board were 20.3cm high while the box measured 36.2cm per side. Four holes, each 1.3cm in diameter, were located on the base of the box large enough for the mouse to poke its head through. The perimeter of the area formed by the outside edge of these four holes formed a square 8.9cm per side, which was outlined with tape. The mice were initially placed in the centre of the box and the test was videotaped for five minutes in a dark room under red light.

Hole Board measures. The hole board apparatus was used to measure mouse activity and exploratory behaviour independently of the plus maze. There were two measures indicative of mouse activity including the number of rears and time spent active in the hole board which was defined as the time the mouse spent mobile. The exploratory tendencies of the mice were categorized into four measures including the frequency of head dips into the four holes, the amount of time spent in the centre of the hole board, and the time spent near the wall. Differentiating between the centre and the walls was done by placing tape on the floor of the hole board connecting the four holes in the centre. The mouse was considered as either in the centre or near the wall depending on which side of

the tape the mouse was located. All four paws had to be in either the central area encompassed by the tape or beyond the tape to be considered in one of the two areas.

Fecal boli were also counted after each test.

Elevated Plus Maze. The elevated plus maze test was conducted immediately after the hole board test. Mice were transferred directly from the hole board to the elevated plus maze and recording resumed. As with the hole board, the test was conducted in a dark room with red lights positioned over the apparatus. This apparatus consisted of four elevated arms, two open and two closed, arranged in the shape of a plus sign. The maze was composed of clear, transparent, Plexiglas with the floor of the four arms painted black in colour. The four arms were 5.1 cm in width and 29.2 cm in length with a 1 cm ridge running along the edge of the open arms, and a 14 cm high clear wall surrounding the closed arms. The edging of the open arm was in place to promote open arm exploration in the maze (Treit et al., 2003). Mice were placed in the centre of the maze facing an open arm of the maze at the start of the test. The test lasted for five minutes and the mice were then returned to their cages and carried to their housing rooms.

Elevated Plus Maze measures. A large number of measures of exploratory tendencies were taken in the plus maze. Both the frequency of open and closed arm entries was measured along with the amount of time spent in the open and closed arms. Entries into a closed or open arm were defined as the mouse having all four paws inside the open or closed arm. The number of head dips and rears were also measured in three

areas of the maze when all four feet were within: the centre, the closed arms (protected) and open arms (unprotected).

A measure of risk assessment was also recorded to further assess mouse behaviour. Risk assessment was scored when the mouse had its hind paws in the closed arm and stretched its head out into an open arm. Frequency of risk assessment was divided by time spent in the closed arms of the maze to give a ratio of risk assessment.

Additional validated measures of anxiety-like behaviour taken in this apparatus were ratio time and ratio entry. These measures have been shown to be sensitive to predator stress and good indicators of the levels of anxiety-like behaviour in rodents (Adamec and Walling, 2004). Ratio time was defined as the time spent in the open arms of the plus maze divided by the total time spent in the open and closed arms combined. Ratio entry was also calculated in the same manner using the number of entries in the open arm, and the total number of entries in any arm. The number of boli present in the open and closed arms of the plus maze was also recorded.

Light Dark Box. Mice were also tested in the light dark box as another measure of anxiety-like behaviour (Bouwknicht and Paylor, 2002). This apparatus consisted of two large chambers connected by a small passageway allowing the mouse to traverse between the two chambers. Each chamber was rectangular in shape measuring 19.1 cm on each side, with walls 14 cm in height. The small rectangular tunnel connecting the chambers measured 6.4 cm high by 7.5 cm wide. The entire apparatus was made of dark grey plastic with a clear Plexiglas cover hinged to the opening on top of one chamber to allow light into the box. This transparent cover also had numerous ventilation holes.

The dark chamber was entirely enclosed with a solid dark grey plastic cover. A 100 watt light bulb was placed 56 cm above the floor of the light chamber and provided illumination at an intensity of 70-foot candles at the floor of the light chamber. Mice were placed in the light chamber at the start of the test and their activity was videotaped for 5 minutes.

Light Dark Box measures. Measures used in the light dark box apparatus included latency to enter the dark chamber at the start of the test, total time spent in both the light and dark chamber, as well as the total number of entries into each chamber. These measures all help quantify the animals' tendency to avoid the light chamber, which has previously been shown to be a good indicator of rodent anxiety-like behaviour (Bouwknicht and Paylor, 2002). The final measures taken were the number of mouse boli in the light and dark chambers.

Cat exposure behavioural measures. Behaviour of both the cat and mouse during the ten-minute exposure was quantified. The mouse behaviours recorded from videotape included frequencies of active, passive, and escape defensive responses to the cat. An active defence was defined as sideways or upright posture, with or without pushing at the cat with a forepaw (Adamec & Walling, 2004). This measure also included any attempts to bite the cat. Passive defences included freezing when the cat approached. An escape was defined as an attempt to leave the immediate area as the cat approached. Each of these measures was recorded separately with respect to the action of the cat that resulted in the defensive behaviour.

Cat behavioural measures included pursuits of the mouse and the frequencies of bites, pawing, and sniffing. Total times were recorded for the amount of time spent by the cat sniffing the mouse as well as time spent near the mouse, which was defined as being within one square block (one foot) of the mouse. Latencies for both sniffing and approaching the mouse were also recorded. A measure of mouse activity was taken by recording the amount of time the mouse spent immobile as well as the number of squares the mouse crossed during the ten-minute exposure. The number of squares crossed by the mouse when the cat was near, in pursuit, or away from the mouse when it crossed the square was also quantified.

Statistical Analysis

Data for the study were analyzed using appropriate Analysis of Variance (ANOVA) design for each behavioural test. The Kruskal Wallis One Way ANOVA design was used when data were not normally distributed. Mean contrasts were done using t tests and Tukey Kramer multiple comparisons of the groups for various behavioural tests or the Kruskal Wallis multiple z test when the Kruskal Wallis ANOVA was employed.

Results

Pre Exposure Startle Response

Pre Exposure peak startle response was assessed with a three-way analysis of variance on groups with repeated measures on startle trial and test day. There was a test day effect only ($F(2, 228) = 17.77, p < .001$). There were significant increases from Day 1, 2, and 3 using Tukey Kramer Mean contrasts $p < .05$ (Figure 1 left panel). However, there were changes in body weight over test days but no group or group \times day effects (Main Test Day effect of Body Weight is shown in Figure 2; ANOVA of Group with repeated measures on Test Day; Test Day Effect ($F(3, 432) = 205.81, p < .001$)). Body weight increased over pre-test days 1 to 3 with a further increase on post treatment test day 4 (Tukey Kramer, $p < .05$). The significant increase in Peak Startle Amplitude from day 2 to 3 was eliminated when body weight increases were controlled by analysis of covariance (Figure 1 right panel; $F(2, 227) = 11.60, p < .001$ Test Day Effect, Tukey Kramer mean contrasts, $p < .05$). This suggests that weight contributed to this difference. However, weight co-variance did not remove the increase in startle amplitude present from day 1 versus day 2 and 3 (Figure 1). Therefore, some sensitization to startle had occurred and stabilized after the first day. This pattern of startle response was consistent over all groups.

Post Exposure Peak Startle Amplitude

An initial analysis was done on peak startle amplitude data to determine whether the Handled and Handled Vehicle groups could be combined as well as the Exposed and

Exposed vehicle groups for subsequent analysis. Four-way ANOVA assessed predator stress effects (handled, cat exposed), injection effects (no injection, vehicle) with repeated measures on startle trial and ambience (light versus dark). The only effect was a main predator stress effect ($F(1, 93) = 4.80, p < .031$, Figure 3). These results indicate that the injection had no effect on startle and permitted combining of injected and uninjected groups within the handled and predator stressed conditions in subsequent analyses.

Due to the non normality of the data (Omnibus Test = 269.54, $p < .001$) further analysis comparing EN101, INVEN101, combined Predator Stressed, and combined Handled Groups used a non-parametric Kruskal Wallis One Way ANOVA on medians. There was a group effect (Group value of $\chi^2(5, n = 180) = 49.54, p < .001$ Figure 4). Predator stress increased startle amplitude over controls except in the group given EN101 where EN101 blocked the startle increase in predator stressed mice, returning levels to those of controls. INVEN101 was without effect on the potentiation of startle by predator stress (Kruskal Wallis multiple comparison z test $p < .05$).

Examination of post exposure peak startle amplitude revealed an habituation like decline only in the light trials (Ambience x Trial, $F(9, 837) = 3.05, p < .002$, Figure 5). Therefore habituation was assessed in light trials alone. Exponential decays were fit to mean startle over trial for handled, exposed (combined), INVEN101 and EN101 groups separately (all df adjusted $r^2 \geq .93$, all $F(2, 9) \geq 44.73, p < .001$, Figure 6). T test analysis of the tau values for the four groups showed that handled mice differed from all other

groups in startle habituation while the combined predator stress group, INVEN101 and EN101 did not differ significantly from each other (all $t(18) \geq 2.38, p < .03$, Figure 7).

Hole Board and Plus Maze Test Results

Preliminary two way analyses of variance assessed predator stress (Handled, cat exposure) and injection (none and vehicle) effects in H, HV and EXP and EXPV groups on hole board behaviour. There were no injection effects or interactions on any measures permitting combining handled (H + HV) and predator stressed (EXP + EXPV) groups. There was a main predator stress effect on one measure in the hole board, time active ($F(1, 96) = 6.16, p < 0.015$). Predator stress decreased time active (Figure 8, top panel).

Combined control and predator stressed groups were then compared to EN101 and INVEN101 groups in a one way ANOVA. Predator stress decreased time active in all groups, but injection of either EN101 or INVEN101 tended to raise activity levels equally to values between combined predator stressed and combined control groups (Figure. 8 bottom panel; Group Effect $F(3, 146) = 2.76, p < .045$; mean contrasts Tukey Kramer, $p < .05$). The behaviour of EN101 and INVEN101 groups cannot be an injection effect alone as EXP and EXPV groups did not differ. However, it is not an effect attributable to EN101 per se either.

To control for possible activity effects on plus maze behaviour, preliminary two way analyses of covariance (time active in the hole board as a covariate) were performed assessing predator stress and injection effects in H, HV, and EXP, EXPV groups on plus maze data. There were no interactions and only one main predator stress effect on ratio entry ($F(1, 95) = 4.06, p < .047$). Predator stress depressed ratio entry (Figure 9).

Predator stress also tended to depress ratio time ($F(1, 95) = 2.47, p < .12$ or $t(95) = 1.72, p < .06$ one tailed). There were no effects on other measures including risk assessment (Figure 9, $F(1, 95) = 1.08, p < .31$). This pattern of findings is consistent with recent studies of the effects of predator stress on anxiety-like behaviour in the plus maze in this strain of mouse (Adamec & Walling, 2004). These data also justified combining controls and predator stressed groups for subsequent comparison to EN101 and INVEN101 groups.

One way analysis of covariance with time active in the hole board as covariate was used to compare combined control and predator stressed and the EN101 and INVEN101 groups on ratio entry. Though the main group effect was not significant ($F(3, 145) = 1.89, p < .14$), in light of predator stress effects in the previous analysis, planned t tests were done on the means of the four groups. This analysis revealed that predator stress reduced ratio entry relative to control and EN101 and INVEN101 did not affect this suppression (Figure 9 bottom panel, mean contrasts $t(145) = 2.352, p < .021$, comparing control to the other three groups which do not differ).

Light Dark Box Results

Pre analyses using two way ANOVA comparing effects of predator stress (handled, cat exposed) and injection (none, vehicle) on light dark box behaviour revealed only predator stress effects and no injection effects or interaction. Main predator stress effects were present in the light dark box for three measures, latency to enter the dark compartment, time in the dark compartment, and time in the light compartment (all $F(1, 96) \geq 11.51, p < .02$). In comparison to handled controls predator stressed mice exhibited

a shorter latency to enter the dark compartment and spent a larger portion of the test in the dark chamber and less time in the light chamber (Figure 10). These analyses also permitted combining H, HIV and EXP, EXPV in subsequent analyses.

One way ANOVA was used to compare combined handled and predator stressed and EN101 and INVEN101 groups on the three measures that had produced significant main predator stress effects. There were main group effects on all measures (all $F(3, 46) \geq 4.52, p < .005$). Mean contrasts (Tukey Kramer, $p < .05$, Figure 11) revealed that predator stress decreased both latency to enter the dark compartment and time spent in the light compartment, and increased time in the dark compartment. EN101 did not alter this pattern of response. In contrast predator stressed mice given INVEN101 fell between controls and the other groups. This is a weak effect and likely requires replication before pursuing.

Cat Test Behaviour Results

There were no group differences in the cat's behaviour or mouse response to the cat on any of the measures taken during the cat exposure. This analysis indicates that any group differences in behaviour post stress are not a result of differential treatment of mice by the cat in the various groups or in the response of the mice to the cat in the different groups.

Discussion

The molecular mechanisms leading to the long-term neuronal hypersensitivity that is characteristic of PTSD are still not fully understood (Meshorer et al., 2001). The goal of this study was to examine the role of the read through variant of acetylcholinesterase in the anxiogenic effects of predatory stress using the predator stress model of PTSD in mice.

EN101 is an antisense oligonucleotide developed by Dr. Soreq in Jerusalem as a therapeutic drug for myasthenia gravis. This drug has been shown to block the mRNA transcription of AChE-R therefore limiting its effect both peripherally and centrally in the brain (Brenner et al., 2003 Pollak et al., 2005). Moreover, EN101 has been shown to selectively inhibit the transcription of the read-through variant of acetylcholinesterase while leaving the normal "synaptic" variant intact (Nijholt et al., 2004). Research indicates that AChE-R over expression is induced under stress in brain regions implicated in PTSD including the hippocampus and amygdala and may play a role in the changes in rodent affect (Cohen et al., 2002, Birikh et al., 2003, Yilmer-Hanke, Roskoden, Zilles, & Schwegler, 2003).

We tested the effects of EN101 on predator stress effects on rodent affective behaviour. Moreover we used INVEN101, the inverse mRNA sequence of EN101 both as a control and to demonstrate the specificity of EN101. This study provides insight into the specific role of AChE-R in changes in rodent affect following stress and lays the groundwork for further research into the potential clinical usefulness of the drug for people suffering from PTSD.

Predator Stress Model

The changes in rodent affect seen in mice following predator stress in this study were consistent with previous research (Adamec et al., 2005, Cohen et al., 2003, and Hage and Belzung, 2002). Mice exposed to a cat exhibited a long-lasting increase in startle amplitude over handled controls, a delay in startle habituation and anxiogenic effects in the plus maze and light dark box.

Effect of EN101 on Behaviour following Predator Stress

Predator stress increased startle amplitude significantly while EN101 blocked this increase, returning startle amplitude to that of controls. This effect is specific in that the anti-sense compound, INVEN101 had no effect on predator-stress enhancement of startle amplitude. These results indicate that EN101 blocked the hyperarousal produced by predator stress in mice. The lack of effect of INVEN101 indicates the specificity of the effects for the blockage of the specific mRNA sequence for AChE-R. Together these data suggest that EN101 reduced the level of AChE-R in brain regions critical to hyperarousal in rodents thereby preventing these changes in behaviour.

Actions of EN101 on effects of predator stress on behaviour were restricted to startle amplitude and were not seen on startle habituation rate. EN101 had no effect on stress-induced anxiety in the light dark box paradigm, though there was a weak effect of INVEN101 in light dark box. Finally both EN101 and INVEN101 increased time active in the hole board to levels in between predator stressed and handled control mice. Since vehicle-injected controls did not show such effects, it is unlikely these are injection

effects. These latter effects are weak and require replication. Importantly there were no effects of these drugs on any of the plus-maze measures or habituation of startle indicating that AChE-R may not play a role in these changes in rodent affect. These findings provide further evidence that behavioural changes observed in startle, elevated plus maze and the light dark box following predator stress are mediated by separable neural substrates (Adamec et al., 1999, Adamec et al., 2006).

Taken together, the results of this study suggest that predator stress abnormally increases the transcription of AChE-R, which in turn alters the neural substrate responsible for the changes in acoustic startle amplitude. EN101, by blocking the mRNA transcription for AChE-R prevents these changes from occurring.

Multiple Neural Systems involved in Anxiogenic Behaviour

Acoustic startle is mediated by simple reflex circuitry in the brain, which has connections in the lower brain stem. Research suggest that fear potentiated startle activation is mediated by neurons in the superior colliculus and the caudate nucleus of the pontine reticular formation which receive heavy input from the amygdala (Davis, 2006, Zhao & Davis, 2004, Yeomans et al., 2006). Cholinergic input from the amygdala originates in the BLA region of the amygdala which projects to the central (CeA) and medial (MeA) nuclei of the amygdala that indirectly project to these regions of the brainstem responsible for acoustic startle response (Davis, 2006).

This lack of a broad-spectrum effect of EN101 across all measures of anxiety-like behaviour in the predator stress model is consistent with a growing body of evidence

indicating different systems are involved in the various changes in rodent affect following predator stress. A variety of neural systems are thought to be involved in PTSD such as the amygdala, hippocampus, and afferents to many other regions in the basal forebrain (Degroot and Nomikos, 2005, Nijholt et al., 2004, and Adamec et al, 2003). Such a plethora of neural circuitry being implicated in the disorder would suggest that no one system could be responsible for such a complex psychological disorder. Moreover, present and past findings like these call into question the idea that different tests represent converging measures of a common substrate of anxiety-like behaviour (Adamec, 2001, Adamec & Blundell, 2003, & Adamec et al., 1999).

In addition, a variety of data implicate neuroplasticity of amygdala efferents to brainstem and hippocampal-amygdala communication, alone or in combination, in stress-induced changes in startle and plus maze but not in the light dark box (Adamec et al., 2005, McIntyre et al., 2003). Increased expression of AChE-R following stress has been demonstrated in some of the same areas involved in predator stress induced limbic neuroplasticity including the hippocampus, amygdala and piriform cortex (Sembulingam et al., 2003, Nijholt et al., 2004 and Sternfeld et al., 2000)

Stressors triggering increased AChE-R include exposure to 30 minutes noise stress and immobilization stress (Sembulingam et al., 2003, Birikh et al., 2003, Nijholt et al., 2004). Nijholt et al., (2004) demonstrated that immobilization stress induces a transient alternative splicing of AChE (AChE-R) in hippocampal neurons. The stratum pyramidale of the hippocampus displays a transient increase in AChE-R which reaches a maximum in 2 hours and returns to baseline within 24 hours. In contrast the stratum

radiatum displayed AChE activity maximal at 24 hours indicating that changes in expression of AChE-R is not purely a transient event (Nijholt et al., 2004). Furthermore, as AChE-R activity returns to baseline levels in the stratum pyramidale it is reaching maximum in the stratum radiatum, which is consistent with the reported stress-induced translocation of AChE-R mRNA from the nucleus of hippocampal CA1 neurons into dendrites (Nijholt et al., 2004). According to Nijholt the physiological relevance of AChE-R is multileveled and it is conceivable that AChE splicing may occur not only in response to stress but also in response to learning itself. Therefore it would be of great interest to determine whether AChE-R participates in sustained maintenance as well as the initiation of stress related changes in affect.

While the hippocampus is the most researched in terms of AChE-R expression, there are other areas involved in neural stress circuitry which display over expression of AChE-R as well, such as the basolateral amygdala (Sternfeld et al., 2000, Birikh et al., 2003). Another molecule that has been linked to AChE-R is the protein kinase C β alternative splicing product PKC β II. Protein kinases (PKC) are known regulators of synaptic transmission and neuronal function and have been shown to play a large role in learning and memory. Research suggests that activation of PKC is necessary for the induction of NMDA receptor-dependent LTP in areas of the hippocampus (Weeber et al., 2000).

The β isoform PKC β II is involved in the translocation of AChE-R within the cells through an interaction with the C terminus of the AChE-R molecule. AChE-R interacts with PKC β II through the scaffold protein RACK1 increasing PKC β II enzymatic activity

and increasing its density in hippocampal neurons (Nijholt et al., 2004, Sklan et al., 2006). The beta isoform of protein kinase C plays a critical role in synaptic plasticity in learning related signal transduction mechanisms in regions of the brain including the hippocampus and basolateral nucleus of the amygdala leading to changes in behavioural affect involved in contextual fear conditioning (Weeber et al., 2000). Weeber et al. (2000) have shown that deletion of the PKC β gene resulted in defects in two amygdala-dependent learning tasks, cued and contextual fear conditioning. Also contextual fear conditioning following stress in rodents is associated with activation of hippocampal PKC and the translocation of PKC β II from the cytosol to the membrane (Nijholt et al., 2004). This body of research is relevant to predator stress in that predator stress potentiates hippocampal efferents to basolateral amygdala shown to be involved in contextual fear conditioning (Adamec et al., 2006).

The link between AChE-R expression and PKC β II is not anatomically uniform, however. Birikh et al., (2003) demonstrated that staining of PKC β II was intensified by stress within several stress-response brain regions in some but not all of the AChE-R accumulating neurons. Neurons in the upper cortical layers, hippocampal CA1 and CA3 regions, the lateral septum, and basolateral amygdala displayed intensified PKC β II staining along with AChE-R accumulation. However AChE-R accumulation neurons in the lateral and ventro-medial hypothalamus, central nucleus of the amygdala, the hippocampal dentate gyrus and the ventro-lateral thalamus showed no PKC β II staining (Birikh et al., 2003). If AChE-R, PKC β II interactions mediate predator stress induced neuroplastic changes underlying some but not all behavioural effects, the co-existence of

these molecules in some but not all regions may explain the selective impact of interference with AChE-R expression on behaviour in the present study.

Conclusions

This study provides strong evidence for a role of the read through variant of acetylcholinesterase (AChE-R) in the etiology of some of the changes in affective behaviour following stress. While this study did not clarify the exact time window during which suppression of transcription of AChE-R with EN101 is effective, it does provide the groundwork for further research.

EN101 did not have a general anxiolytic effect on all measures of anxiety-like behaviour in the predator stress model of PTSD, suggesting that changes in AChE-R expression are not the only neural substrate involved in the alterations of affective behaviour. While this is congruent with previous research it reaffirms the importance of continued research into the neural mechanisms underlying the various facets of response to traumatic stress. It should be acknowledged that the effects of EN101 on acoustic startle may be a result of non-specific peripheral effects of the drug. While this is unlikely it cannot be ruled out in the present study.

The concept of various neural systems involved in the precipitation of various changes in affective behaviour following traumatic stress is consistent with the fact that a diverse group of anxiolytic drugs are currently in use which act on a number of different neural substrates including the cholinergic, serotonergic, and noradrenergic systems in the brain (Moralik et al., 2005, Degroot & Nomikos, 2005, Gulpinar & Yegen, 2004, Kaufer et al., 1998, McEwen, 1998). In addition there are a variety of pharmacological

compounds acting on different neural substrates, which in turn exhibit varying degrees of success in treating different symptoms of PTSD. Treatments currently being used to treat these conditions include forms of psychotherapy such as cognitive behavioural therapy, pharmacological treatments such as selective serotonin re-uptake inhibitors (SSRI's), monoamine oxidase inhibitors (MAOIs), noradrenergic beta-blockers, and benzodiazepines (Degroot and Nomikos, 2005, Vaiva et al., 2003, Ballenger, 1999). While there is no one type of treatment which offers a "cure" for this disorder, the treatments listed above do provide relief from some of the debilitating symptoms experienced by people suffering from this disorder.

While this study is preliminary in nature, it provides evidence for the potential use of EN101 in the treatment of PTSD and its symptoms. Further research is necessary to determine the exact window during which the drug should be administered in order to obtain the greatest efficacy. In this study, we administered the drug at multiple time intervals before and after the stressor occurred. We also did not include a EN101 non stress control group despite the potential for EN101 to have an effect on behaviour in the absence of stress. This decision was based on the limited supply of the drug; the fact that there have been no reports of general effects of the drug; and the fact that behavioural tests occurred after the drug effects would have worn off. Future research will help delineate the effectiveness of this drug in altering levels of AChE-R following stress and subsequently possibly diminishing changes in affective behaviour such as hypervigilance in people suffering from PTSD.

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Figure Legends

Figure 1

Plotted in this figure are the means + SEM of peak startle amplitude in volts for the pre startle response over test days. The left portion of this graph displays the values for the raw startle data, while the right side displays the same data covarying weight. Test day means marked with the same letter do not differ, while means that do differ are marked with different letters ($p < .05$).

Figure 2

Plotted in this figure is mean + SEM body weight in grams over pre-exposure startle test days. Comparisons made between the means for the four test days are labeled as in Figure 1.

Figure 3

Plotted in this figure are the mean + SEM peak startle amplitudes in volts of the post cat exposure startle for handled versus predator stressed groups. Comparisons made between the means for the two groups are labeled as in Figure 1.

Figure 4

Plotted in this figure are median + SEM peak startle amplitudes in volts for the post cat exposure startle for handled, predator stressed, EN101 and INVEN101 groups.

Comparisons made between medians are labeled as in Figure 1.

Figure 5

Plotted in this figure are peak startle amplitudes in volts (mean \pm SEM) for post exposure startle over all groups plotted for each trial for the light (open squares) and dark startle (dark, filled squares) separately. A "Dt1" indicates that the dark trial differs from Dark Trial 1. A "Lt2,3" indicates that the light trial differs from Light Trials 2 and 3. A "#" indicates that the dark trial differs from the corresponding light trial (all $p < .05$ Tukey Kramer Test).

Figure 6

Plotted in this figure are peak startle amplitudes in volts for post stress light startle trials only. Exponential decays were fit to means over trial blocks for handled, exposed, INVEN101 and EN101. Plotted are the raw data, exponential fit and smoothed data FFT 15% as per the legend.

Figure 7

Plotted in this figure are the tau(τ) values (+ SE) for startle habituation for the handled, predator stressed, INVEN101 and EN101 groups. A "#" indicates that the handled group differed significantly from all others, which did not differ.

Figure 8 (top panel)

Plotted in this figure are mean (+ SEM) time active values in seconds in the hole board test for combined control and combined predator stressed groups. Comparisons made between the means for the two groups are labeled as in Figure 1.

Figure 8 (bottom panel)

Plotted in this figure are mean (+ SEM) time active values in seconds for the hole board test for four groups; combined handled, combined predator stressed, EN101 and INVEN101. Means marked with the same letter do not differ. Means marked with different letters differ. Means marked with two letters fall between means bearing either of those letters.

Figure 9 (top panel)

Plotted in this figure are mean + SEM ratio entry, ratio time, and ratio frequency risk (Rfrisk) for control and predator stressed groups covarying time active from the hole-board test. Comparisons made between the means for the two groups are labeled as in Figure 1.

Figure 9 (bottom panel)

Plotted in this figure is the ratio entry covarying time active as in the top panel for all four groups (combined control, combined predator stressed, EN101 and INVEN101). Comparisons made between the means for the four groups are labeled as in Figure 1.

Figure 10

Plotted in this figure are mean + SEM of latency to enter the dark compartment, time spent in the dark compartment and the time spent in the light compartment respectively in seconds for the control and predator stressed groups. A “#” indicates that the combined (EXP, EXPV) predator stressed groups differed significantly from the combined (H, HIV) control groups on that measure.

Figure 11

Plotted in this figure are mean + SEM of measures in light dark box in seconds for all four groups (control, predator stressed, EN101 and INVEN101). Comparisons made between the means for the four groups are labeled as in Figure 8 (bottom panel).

Figures

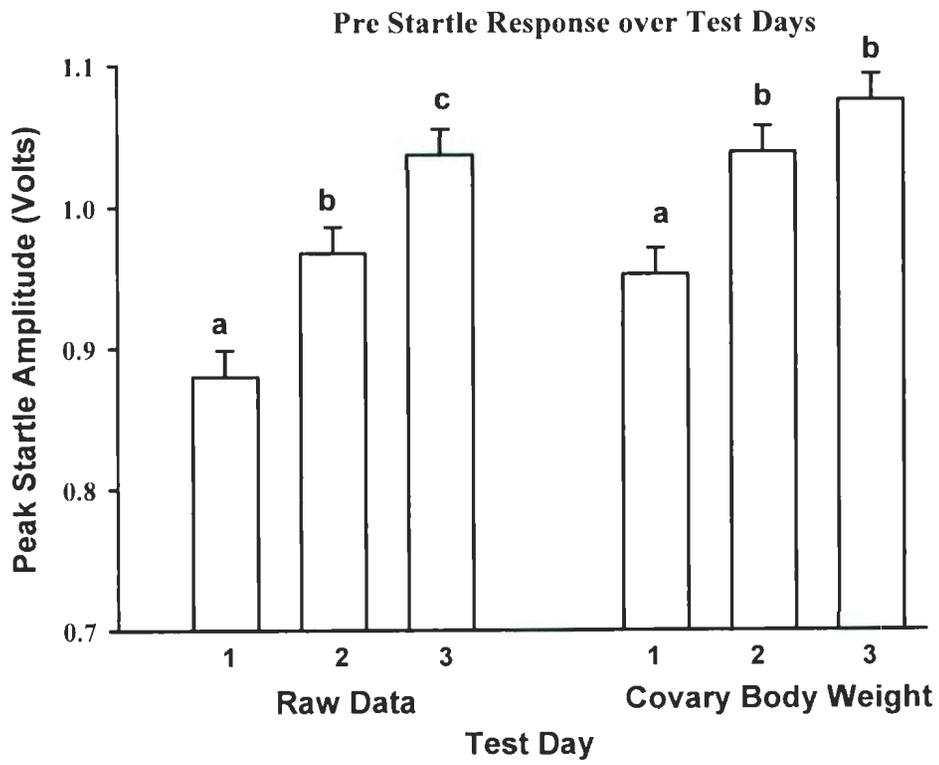


Figure 1

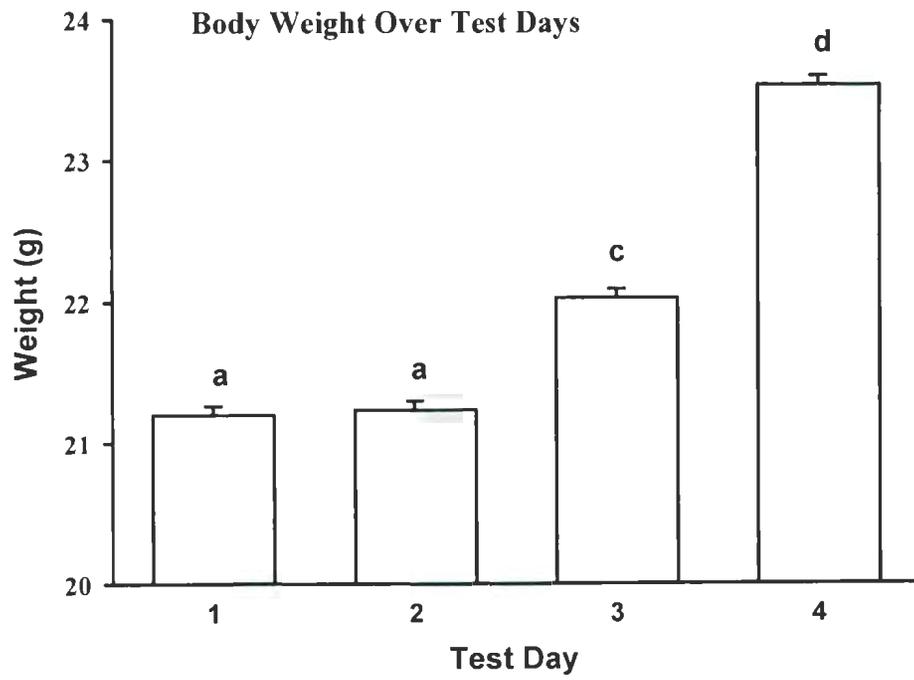


Figure 2

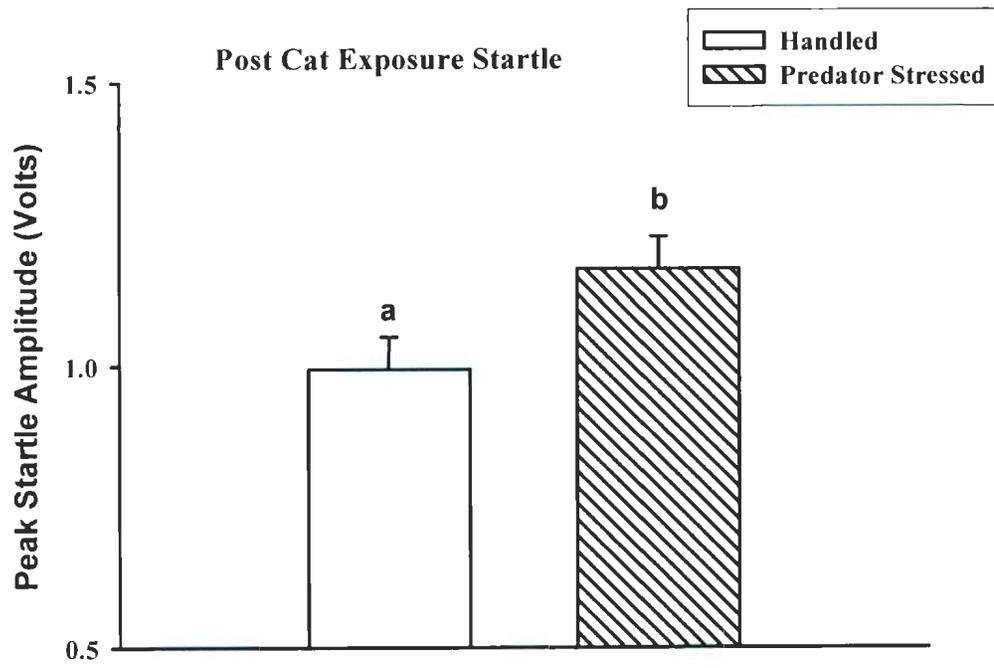


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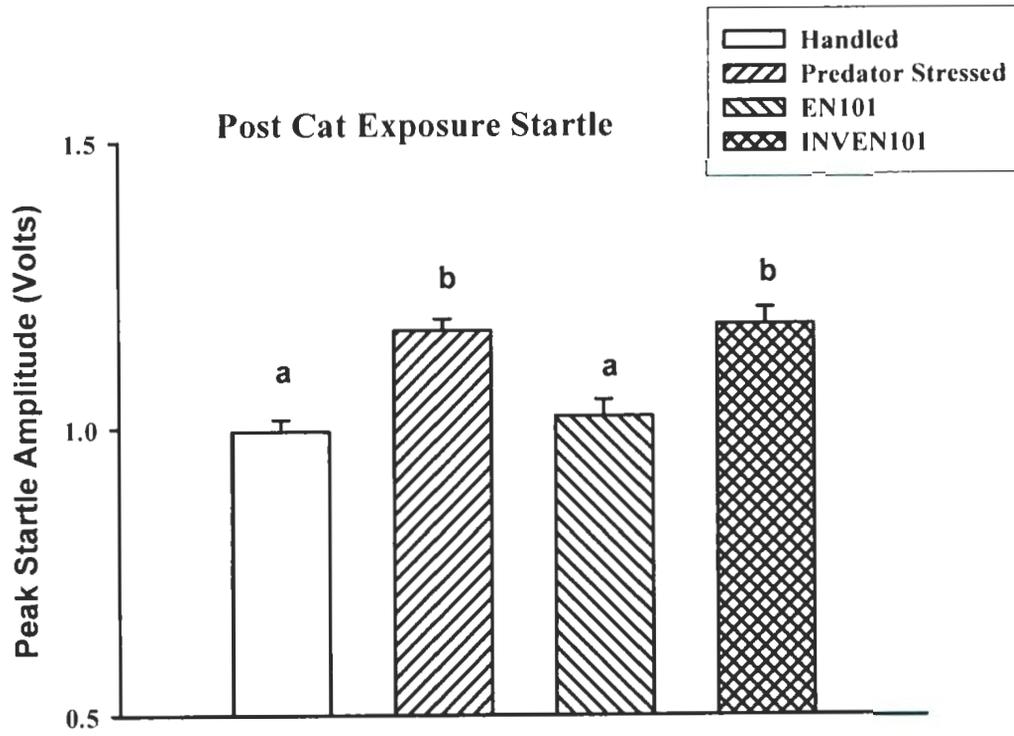


Figure 4

Post Exposure Startle Over all Groups (combined H, E, EN101, INVEN101) Light Dark x Trial

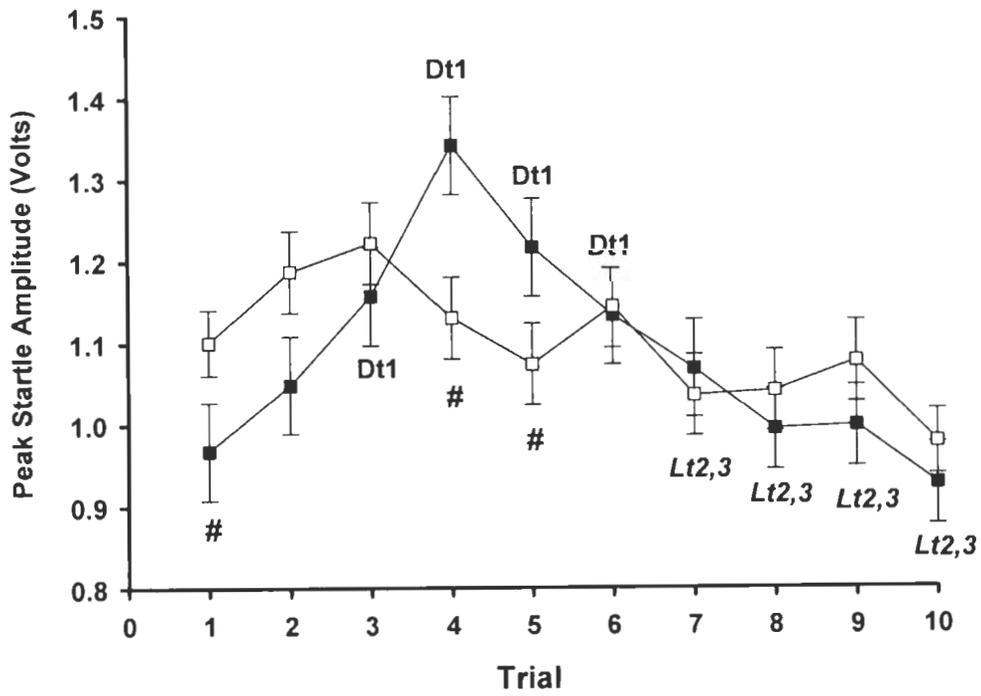


Figure 5

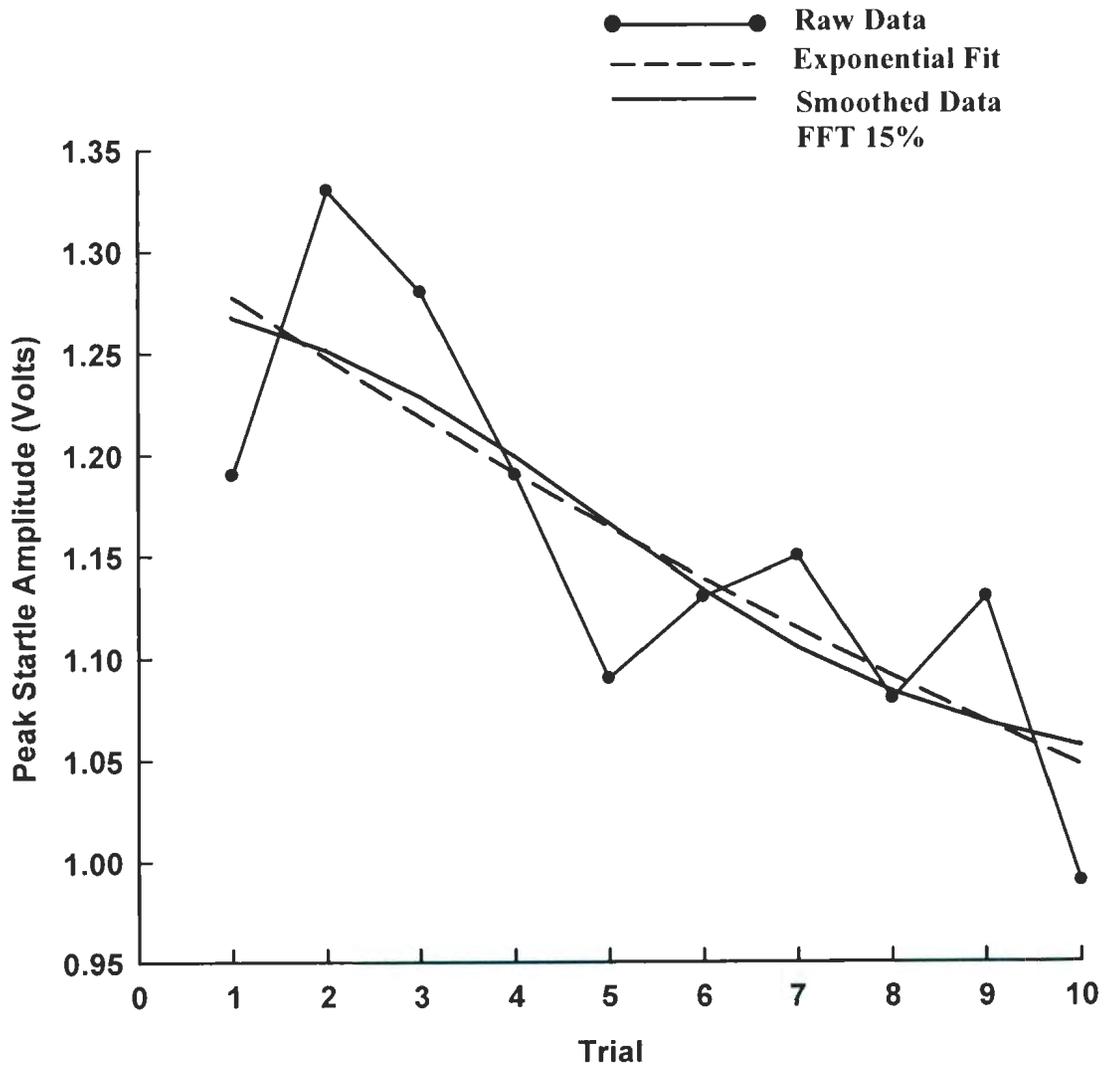


Figure 6

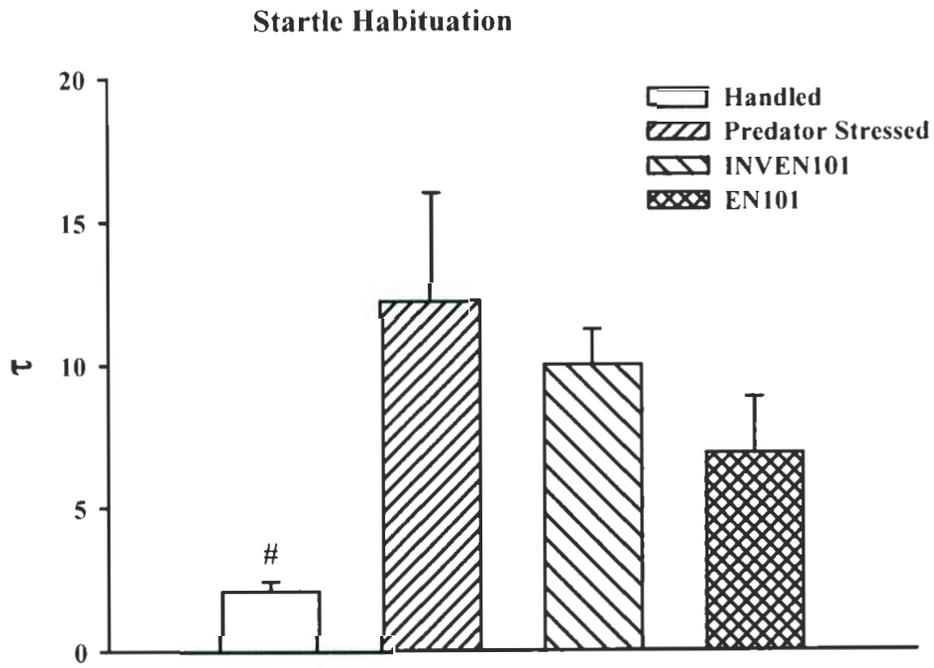


Figure 7

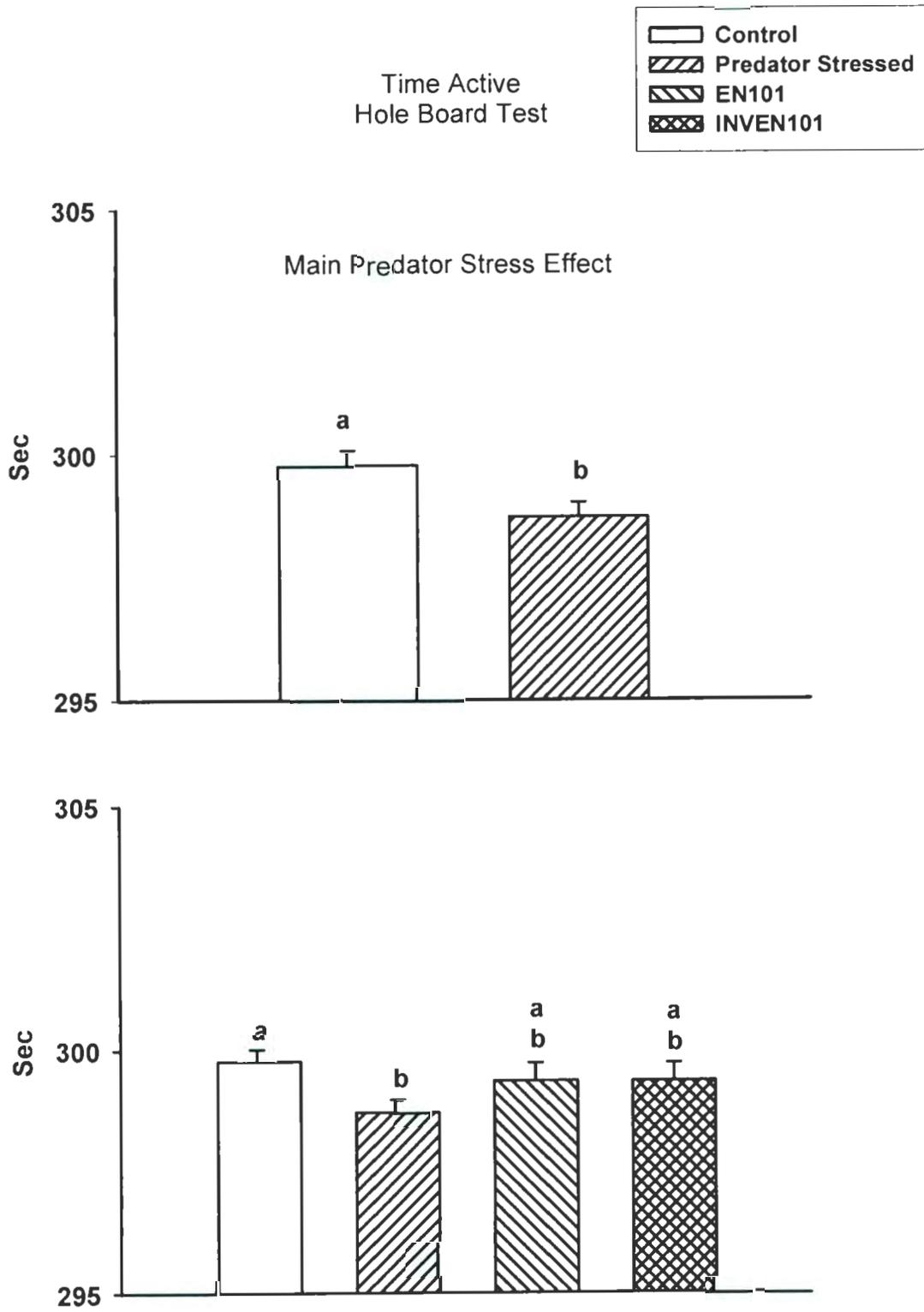


Figure 8

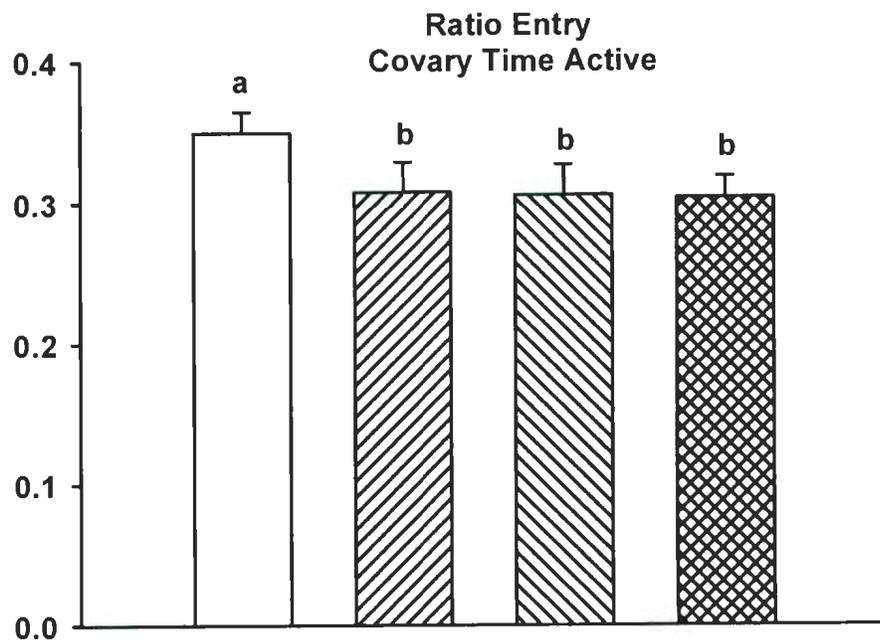
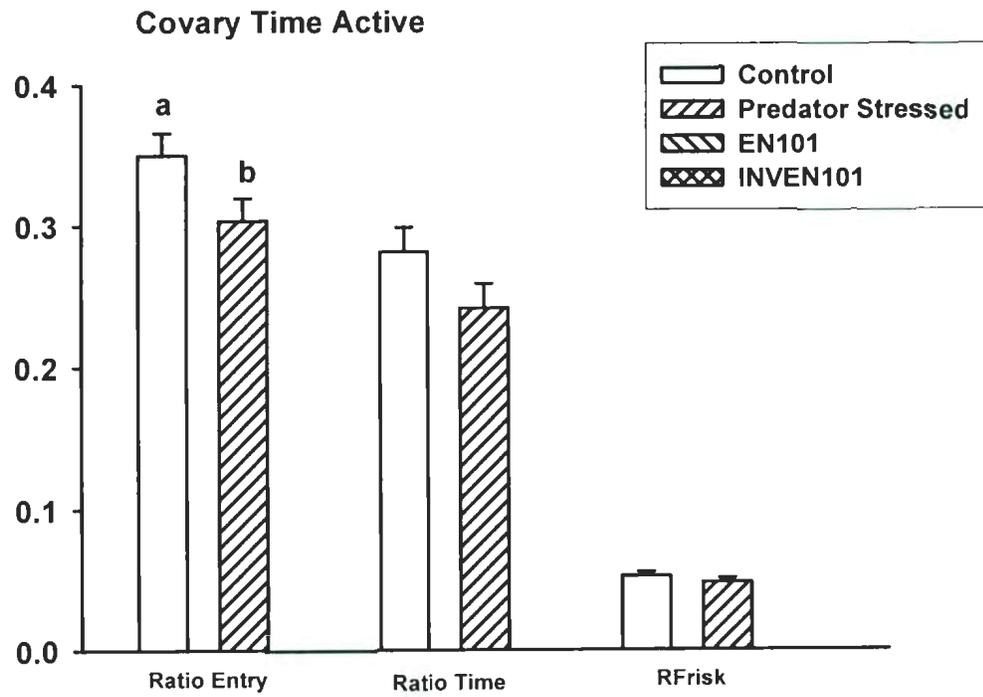


Figure 9

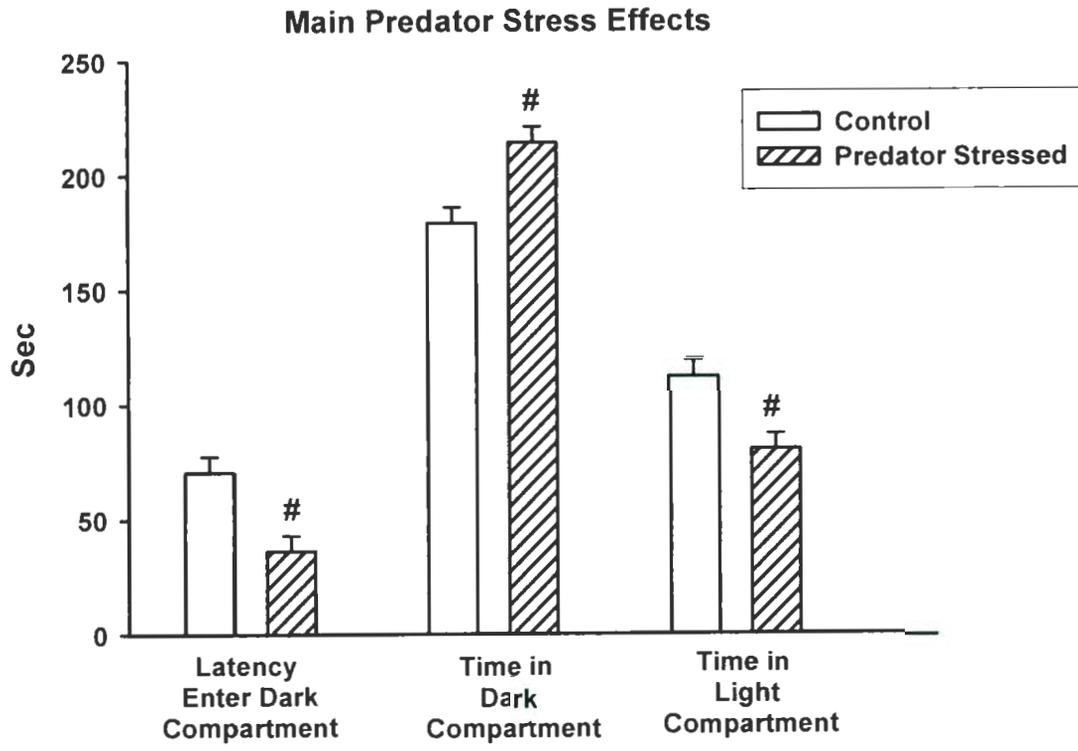


Figure 10

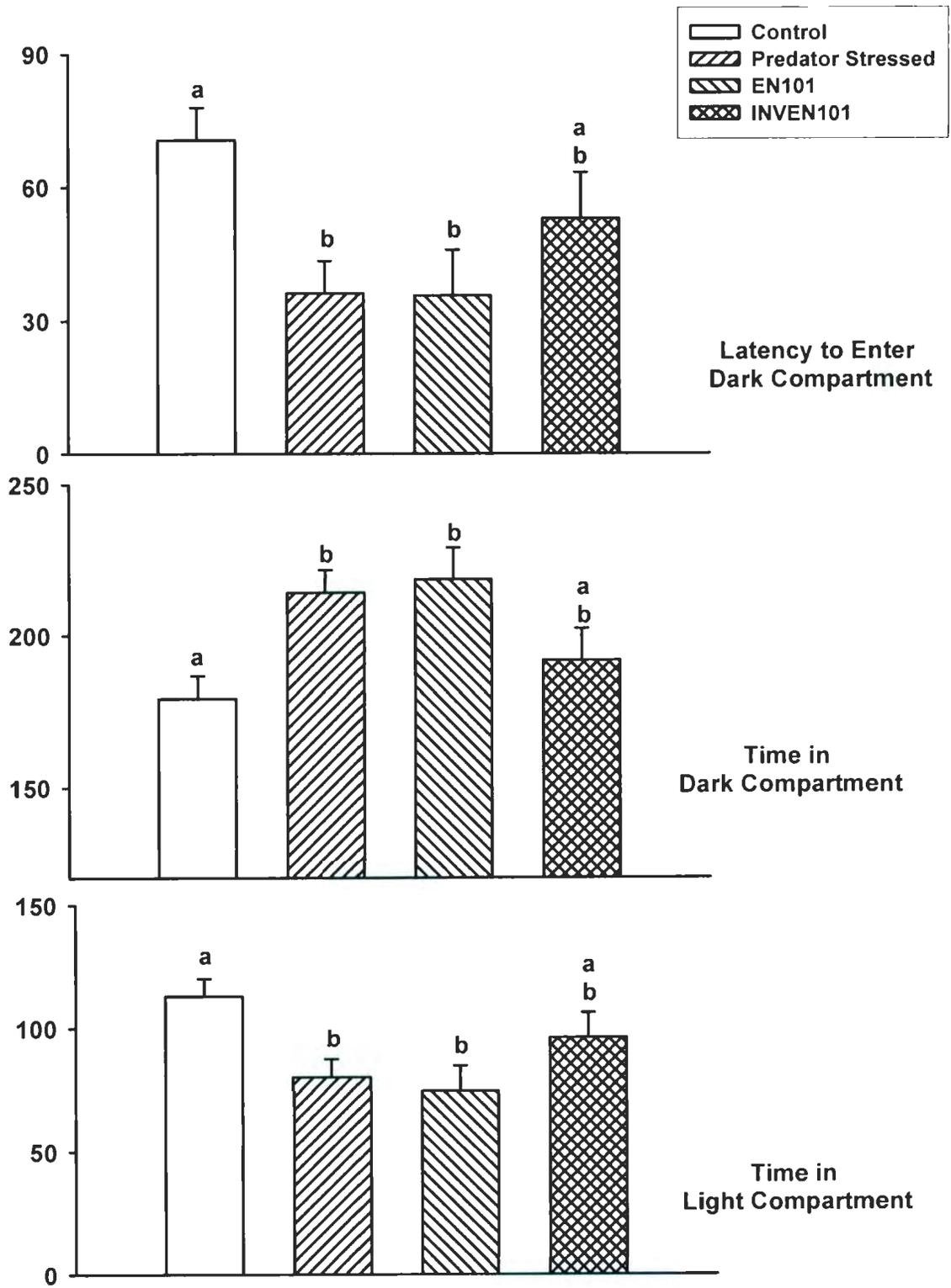


Figure 11

