BEHAVIORAL SENSITIZATION EFFECTED BY VARIABLE ANXIOGENIC CHALLENGE AND PSYCHOGENIC STRESSOR EXPOSURE IN ANXIETY AND MOTIVATIONAL PARADIGMS IN CD-1 MICE: IN SITU HYBRIDIZATION AND IMMUNOHISTOCHEMICAL DETERMINATIONS IN SELECTIVE MESOCORTICOLIMBIC SITES

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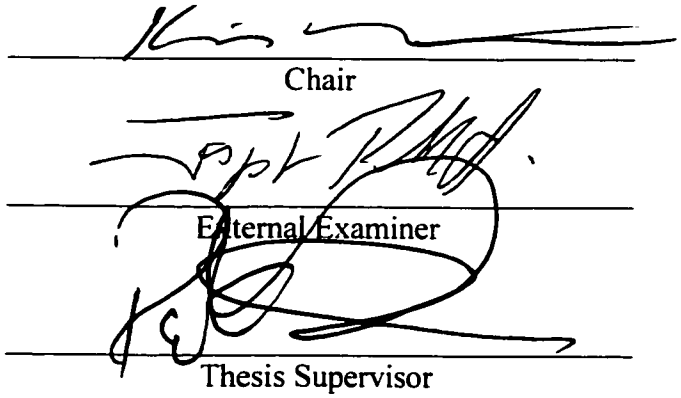

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GENERAL ABSTRACT

It is well documented that stressful life experiences contribute to the etiology of human mood disorders. Arguments for behavioural and neurochemical conditioning and sensitization have been proposed in documentation pertaining to the vulnerability of clinical populations to relapse, the history of such relapses and symptom exacerbation over protracted intervals. A role for cholecystokinin (CCK) in the induction and persistence of anxiety and major depression in infrahuman and human subjects appears to be conspicuous. While increased cerebrospinal (CSF) CCK has been associated with motivational loss, anticipatory anxiety and panic increased enkephalin (ENK) availability in humans has been associated with coping and mood elevation. The present series of experiments provide behavioral evidence for a CCK-ENK interface in the modulation of anxiety and motivation following psychogenic and pharmacological stressor applications. In particular, exposure of CD-1 mice to predator odors increased anxiety in the light-dark box immediately following odor exposure. Anxiety was associated with increased CCK mRNA and decreased ENK mRNA from sub-areas of the amygdala and nucleus accumbens. These changes in CCK and ENK gene expression were transient and were not associated with protracted levels of anxiety. In contrast, repeated exposure of CD-1 mice to the startle stimulus following varying durations of predator exposure resulted in an exaggeration of startle reactivity for up to one week following odor exposure. In contrast to enhanced anxiety in the light-dark box and startle paradigms, predator exposure did not influence reward thresholds among mice responding for brain stimulation from the dorsal aspects of the VTA. The experimental parameters underlying the manifestation of fear and anxiety in CD-1 mice following psychogenic stressor challenge were paralleled by pharmacological assessment of the anxiogenic and anxiolytic influence of central CCK and enkephalin administration, respectively, within these identical paradigms. In pharmacological investigations, the sensitizing effect of a previous anxiogenic dose of CCK-8 sulfated (CCK-8S) to a CCK challenge dose was blocked by coactivation of μ and δ receptors by the enkephalin agonist DALA in an ICSS but not an exploration paradigm among CD-1 mice. Interestingly, administration of intraventricular CCK-8S or systemic Boc CCK-4 increased acoustic startle in mice dependent upon the stressor history of the animal. These data parallel other studies evaluating the propensity of traditional anxiolytic and antidepressant agents to counteract the sensitization process. Evidently, the light dark task, acoustic startle, ICSS and exploration paradigms appear to be differentially sensitive to diverse stressor applications and site specific alterations in mesocorticolimbic ENK and CCK may underlie the expression of anxiety-like behavior within these paradigms. In effect, while CCK induces relatively protracted behavioral disturbances in both infrahuman and human subjects following stressor applications, μ/δ receptor activation may change the course of psychopathology. Potential anxiogenic and mnemonic influences of mesocorticolimbic CCK and ENK availability as well as the time course and underlying neuronal substrates of long-term behavioral disturbances (i.e., behavioral sensitization) as a result of stressor manipulations are discussed.

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EXPERIMENTS 1 & 2

ABSTRACT

Exposure of CD-1 mice to a novel environment as well as exposure to a novel environment lined with soiled rat shavings induced anxiety in the light dark box. Mice exposed to the novel environment (CO) or rat odor (PO) displayed decreased latency to enter the dark chamber of the light dark box and spent less time in the light portion of the apparatus relative to home-caged (HC) mice. These behavioral deficits were still in evidence 4 hours following stressor presentation. Mice exposed to the novel environment or predator odor displayed elevated CCK mRNA levels from the medial prefrontal cortex (mPFC), ventral tegmental area (VTA), medial (MEA) and basolateral (BLA) nuclei of the amygdala relative to home-caged mice. Mice exposed to rat odor displayed elevated mPFC and VTA CCK mRNA relative to mice exposed merely to the novel environment 1 hour post-stressor. In contrast, mice merely exposed to the novel environment displayed elevated BLA CCK mRNA relative to mice exposed to rat odor 30 minutes post-stressor. There were no differences in CCK mRNA values in the MEA among mice exposed to the novel environment or rat odor. In the acoustic startle paradigm, exposure of CD-1 mice to 2, 5 or 10 minutes of rat odor increased acoustic startle relative to mice merely exposed to the novel environment or home-caged mice at protracted intervals. Mice exposed to the novel environment did not exhibit enhanced startle relative to home-caged mice at any of the test intervals examined. Exaggerated startle reactivity was in evidence immediately, 24, 48 and 168 hours following a 5 minute exposure of mice to rat odor relative to mice in the 2 control conditions. In contrast, a 10 minute exposure of mice to rat odor produced an oscillating pattern of enhanced startle evident during the immediate and 48 hour post-stressor intervals only. However, when the startle stimulus was withheld 30 or 60 minutes following odor presentation on the immediate day, mice exhibited enhanced startle patterns reminiscent of the 5-minute exposure. The 2 minute exposure of mice to rat odor produced a delayed onset of enhanced startle observed at the 168 hour test interval only. Potential anxiogenic influences of mesocorticolimbic CCK availability as well as the time course and underlying neuronal substrates of long-term behavioral disturbances as a result of stressor manipulations are discussed.

EXPERIMENT 3

ABSTRACT

Acute exposure of CD-1 mice to the novel odor, butyric acid or the predator odor, 2,5-Dihydro-2, 4,5-trimethylthiazoline (TMT) the major component of the anal gland secretions of the red fox, induced a variety of risk assessment behaviors, suppressed non-defensive behaviors, induced anxiety in the light dark paradigm and increased mesocorticolimbic CCK mRNA immediately following odor presentation relative to saline treated mice. In brief, mice exposed to butyric acid or TMT contacted the odorant cloth less, displayed increased defensive burying, increased frequency of stretch attends and decreased rearing behavior relative to mice exposed to saline. Only mice exposed to TMT displayed freezing. In the light-dark paradigm, mice exposed to either butyric acid or TMT took longer to reenter the light section of the apparatus and spent less cumulative time in the light relative to mice exposed to saline. During the second 5 minute but not the first 5 minute segment of light dark box behavioral analysis, mice exposed to predator odor spent less time in the lit portion of the apparatus relative to mice exposed to either saline or butyric acid. Mice exposed to TMT displayed decreased light and dark compartment transitions relative to mice exposed to butyric acid or saline. Mere odor presentation was associated with a gradual emergence of increased CCK mRNA in mesocortical sites. Butyric acid incited enhanced CCK gene expression in the VTA while both butyric acid and TMT increased mPFC CCK mRNA levels relative to saline treated mice. Increased CCK mRNA within the VTA and mPFC was not associated with anxiety in the light-dark paradigm in CD-1 mice. In contrast, anxiety in the light-dark paradigm evoked increased CCK mRNA expression in the basolateral nucleus of the amygdala in mice exposed to butyric acid or TMT. The relative neurochemical sensitivity of select mesocorticolimbic sites to psychogenic stressor manipulations and the provocation of anxiety-like behavior associated with such experiences is discussed.

EXPERIMENTS 4, 5 & 6

ABSTRACT

Acute psychogenic stressor encounter among CD-1 mice has been associated with behavioral and mesolimbic signatures immediately following aversive life experience. Such behavioral perturbations induce significant yet transient neuronal activation (i.e., fos related antigens) in discrete mesolimbic sites. Naïve, handled CD-1 mice were exposed to the scent of a fox (TMT; threat odor), butyric acid (BA; novel odor) or saline (SAL; neutral odor) in the home cage. Defensive response styles accompanying such exposure were encoded and mice were immediately transferred to the light-dark task, acoustic startle or brain stimulation (ICSS) paradigms. The procedures were evaluated 24, 48 and 168 hours following initial odor encounters in independent groups of mice in the light-dark box or within subjects in the startle and ICSS paradigms. Sub-areas of the nucleus accumbens, amygdala, VTA and medial prefrontal cortex (mPFC) were assessed for Fos-related antigen (FRA), enkephalin mRNA (ENK) and neuronal activation of ENK neurons (FRA/ENK). Mice exposed to TMT made less contacts with the odorant cloth, exhibited decreased rearing and grooming activity and displayed enhanced defensive burying followed by heightened levels of freezing relative to mice exposed to BA or SAL. Exposure of mice to TMT increased FRA in the mPFC, VTA and core and shell of the nucleus accumbens relative to BA or SAL treated mice. The immediate test session hosted the most robust increase in FRA. Within the core and shell of the nucleus accumbens exposure of mice to either BA or TMT increased FRA/ENK neuronal activation immediately. Among mice exposed to TMT, freezing scores were associated with enhanced FRA and ENK/FRA from the shell of the nucleus accumbens and decreased ENK mRNA from the central amygdaloid nucleus. In the light-dark task, mice exposed to BA or TMT demonstrated increased (a) light chamber latency reentry and (b) decreased cumulative time in light relative to SAL treated mice. Only mice exposed to TMT displayed reduced inter-compartmental transition frequency. Among mice exposed to TMT and not BA, anxiety in the light-dark box was associated with increased FRA and decreased ENK mRNA in the accumbal shell and core, respectively. Anxious mice as defined by the cumulative time spent in the light-dark box displayed increased ENK mRNA in the basolateral (BLA), central (CEA) and medial (MEA) amygdaloid nuclei relative to their non-anxious counterparts. In the BLA and CEA, anxious mice also displayed increased FRA/ENK. In the startle paradigm, mice exposed to BA exhibited a startle profile reminiscent of SAL treated mice. A 2 minute exposure of mature mice to TMT enhanced acoustic startle immediately, 24 and 48 hours while a 10 minute session increased startle 168 hours post-odor exposure. In contrast, exposure of mice to BA or TMT for 10 minutes was ineffective in reducing rewarding brain stimulation performance (ICSS) from the dorsal aspects of the VTA. Evidently, the light dark task, acoustic startle and ICSS paradigms appear to be differentially sensitive to diverse aspects of anxiety and may underlie site specific alterations in mesocorticolimbic ENK.

EXPERIMENT 7

ABSTRACT

The influence of systemic Boc CCK-4 (0, 5 μg and 15 μg) or intraventricular CCK-8S (0 and 50 ng) on startle amplitude, startle duration and latency of startle onset among CD-1 mice was evaluated in the fear potentiated startle paradigm immediately, 24 hours, 48 hours and 168 hours following anxiogenic drug administration. Among Boc CCK-4 treated mice, exposure to a conditioned stimulus, light previously paired with footshock, coterminating with a tone (startle stimulus) enhanced startle amplitudes and decreased startle latency relative to Tone alone 168 hours post-drug injections. Systemic administration of 0 and 5 μg did not influence startle potentiation, whereas 15 μg Boc CCK-4 abolished the potentiating effect. Among CCK-8S treated mice, exposure to conditioned stimulus also enhanced startle amplitudes relative to Tone alone. CCK-8S increased startle amplitude 48 hours and 168 hours post-drug administration irrespective of prior stressor history. Mice in the Light + Tone condition exhibited increased latency to startle onset relative to mice in the Tone condition in the immediate post-drug interval only. CCK-8S decreased startle duration in the Tone condition of previously shocked mice immediately, 24 hours and 168 hours post-drug administration relative to saline treated mice. Among non-shocked mice, CCK-8S increased startle duration in the Tone condition immediately, 24 hours and 168 hours post-drug administration relative to saline treated mice. CCK-8S decreased and increased startle duration in the Light + Tone condition 48 hours and 168 hours post-drug administration relative to saline treated mice, respectively. Potential anxiogenic and mnemonic influences of central CCK_B receptor activation in the amygdala and mesolimbic projection sites are discussed.

EXPERIMENTS 8 & 9

ABSTRACT

The proactive influence of intraventricular administration of the mixed μ/δ-receptor enkephalin agonist D-Ala^2-Met^5-enkephalinamide (DALA) (1.0 μg) following central cholecystokinin-8S (CCK-8S) (50 ng) application was evaluated among CD-1 mice to a challenge dose of CCK-8S (5 ng). Cholecystokinin administration decreased responding for brain stimulation immediately from the VTA as well as locomotor and rearing behavior in an exploration paradigm among independent groups of animals. CCK decreased responding for previously rewarding brain stimulation (ICSS) from the dorsal (but not the ventral) aspects of the VTA during the immediate and 24-hour post-stressor intervals. CCK decreased locomotor activity for 168 hours following initial drug injection. CCK decreased rearing behavior during the immediate post-stressor interval only. DALA was ineffective in abrogating CCK-induced ICSS deficits in animals responding for brain stimulation from the dorsal aspects of the VTA during the immediate post-stressor interval. However, DALA restored ICSS to baseline values 24 hours following CCK/DALA administration. DALA enhanced locomotor and rearing scores of CCK treated mice (CCK/DALA) relative to saline (CCK/SAL) treated mice following CCK administration and relative to baseline values (SAL/SAL) immediately post-stressor but not relative to SAL/DALA treated mice. Twenty-four and 48 hours following CCK/DALA administration locomotor scores were depressed relative to SAL/DALA and SAL/SAL treated mice. SAL/DALA treated mice exhibited elevated locomotor scores relative to mice in the SAL/SAL drug condition immediately post drug injections. Rearing scores were depressed among CCK/DALA treated mice 24, 48 and 168 hours relative to SAL/DALA and SAL/SAL treated mice. DALA ameliorated the deficits induced by central CCK administration during the immediate post-stressor interval only and rearing scores of CCK/DALA mice were indistinguishable from CCK/SAL and SAL/SAL treated mice 24, 48 and 168 following drug applications.

Eighteen days following initial CCK-8S and μ/δ challenge, mice were reexposed to a previously established sterile dose of CCK-8S. The 1.0 μg dose of DALA increased ICSS and locomotor activity yet decreased rearing behavior in CCK treated mice challenged to 5 ng dose of CCK (CCK/DALA/CCK) compared to an intervening dose of SAL (CCK/SAL/CCK). ICSS behavior was decreased in CCK/SAL/CCK treated mice during the immediate and 24 hour test session relative to CCK/DALA/CCK and SAL/SAL/SAL treated mice. ICSS patterns of mice in the CCK/DALA/CCK group were elevated relative to SAL/SAL/SAL and SAL/DALA/CCK 48 hours post CCK reexposure. Locomotor activity was elevated in CCK/DALA treated mice relative to CCK/SAL treated mice reexposed to either SAL or CCK during the immediate post-drug reexposure test session. Rearing activity was decreased in CCK/DALA treated mice relative to CCK/SAL treated mice reexposed to either SAL or CCK during the immediate post-drug reexposure test session. Locomotor and rearing behavior was decreased in SAL/DALA treated mice reexposed to CCK relative to SAL/SAL/SAL mice. SAL/SAL/CCK treated mice exhibited reduced rearing scores relative to SAL/SAL/SAL mice during the immediate, 24 and 48 hour test session. These data imply that while CCK induces relatively protracted behavioral disturbances, μ/δ receptor activation may change the course of psychopathology. The neural mechanisms that subserve ICSS are not congruent with those that underlie expression of exploratory behavior.

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EXPERIMENT 4

GENERAL INTRODUCTION

The putative influence of aversive life events to the provocation, maintenance and exacerbation of psychological disturbance is well documented (Anisman & Zacharko, 1990; Anisman & Zacharko, 1992; Bremner, 1999; Brier, 1989; Cui & Vaillant, 1997; Kessing et al., 1998; Kim & Yoon, 1998; Loas, 1996; Mazure, 1994; Risch, 1997; Weiss et al., 1999). The notion that stressful life events provoke or exacerbate psychopathology in humans is appealing. Indeed, among infrahuman subjects, exposure to stressors induces protracted neurochemical variations in selective mesolimbic sites (Cabib & Puglisi-Allegra, 1996; Cabib et al., 1998), which may sustain psychological disturbance (Lovibond, 1998; Parker et al., 1998; Weiss et al., 1999) and increase vulnerability (Drevets et al., 1998; Graham et al., 1999) to ensuing stressor encounter. Retrospective and prospective investigations suggest that affective reactivity to stressful life events is predicated on early life experiences (Anderson, 1999; Brown, 1993), available coping strategies (Bedi, 1999; Biondi & Picardi, 1996; Brier, 1989; Haeri et al., 1996; Husaini, 1997; Kashini et al., 1999), premorbid personality variables (Garnefski et al., 1990; Manfro et al., 1996) and genetic predisposition (Agid et al., 1999; Biondi & Picardi, 1999; Kendler et al., 1999; Klein, 1999).

Infrahuman investigations reveal that in addition to organismic and experiential variables, the stressor experience per se influences long term behavioural and neurochemical alterations attending stressor encounter. Indeed, the severity and duration of aversive stimulation appear to influence the nature and the persistence of region specific neurochemical alteration. For example, mild stressor experiences alter mesolimbic rather than nigrostriatal dopamine (DA) availability (Deutch et al., 1985, 1990, and 1991; Horger & Roth, 1996). Indeed, in vivo microdialysis revealed that a psychological stressor (e.g., auditory, visual and

olfactory cues of conspecifics exposed to footshock) increased DA levels in the shell of the nucleus accumbens for 2 hours following stressor termination in rats, while DA levels in the nigrostriatal core of the nucleus accumbens were unaffected (Wu et al., 1999). In a similar vein, 6-OHDA induced mesocortical DA depletion potentiated the increase of extracellular DA in the shell but not the core of the nucleus accumbens to a d-amphetamine dose approximating the neurochemical consequences of mild stressor exposure (Heidbreder & Feldon, 1998; King et al., 1997). Excitotoxic lesions of the nucleus accumbens shell but not the core produced hypoactivity and attenuated locomotor activity to an ascending d-amphetamine dose regimen in rats (Parkinson et al., 1999). Moreover, excitotoxic and neurotoxic lesions of the central and lateral amygdaloid nuclei attenuate conditioned freezing in rats to cues previously paired with footshock (Goosens & Maren, 2001; Wallace & Rosen, 2001). Finally, mesocortical DA depletion augments anxiety in the elevated plus maze in rats (Espejo, 1997). Indeed, mild stressors alter exploration (Miczek et al., 1999), reduce palatable food consumption (Di Chiara et al., 1999), increase light-dark box anxiety (MacNeil et al., 1997) and elicit defensive response styles among rats and mice (Blanchard et al., 1997; Blanchard et al., 1990; Kemble & Bolwahnn, 1997) in resident/intruder, tail pinch, abbreviated footshock and predator odor paradigms respectively.

It should be underscored that qualitatively defined (e.g., mild) and neurochemically selective (e.g., mesolimbic DA) stressor types are ambiguous. In particular, stressor severity operationalized along parametric characteristics may delineate stressor impact, yet inter-stressor severity comparisons appear to rely on subjective assessment. Such comparative analyses often extend to neurogenic and psychogenic stressors. For example, exposure of animals to 2 minutes of 1.5 mA footshock is considered milder than a 2 minute 2.5 mA

footshock regimen or 60 minutes of the identical stressor. Similarly, 2 minutes of predator exposure appears mild relative to a 60 minute predator encounter. Moreover, 2 minutes of restraint or immobilization has been offered as an example of a milder stressor than 2 minutes of 1.5 mA footshock. Likewise, a 5 minute elevated plus maze experience would be considered a milder stressor than 5 minutes of predator exposure. Yet, it is not clear whether mild stressors represent the minimal stressor intensity sufficient to effect specific neurochemical change in a particular brain site or to elicit a particular behavioural effect. Such stressor definitions are not trivial since, at the very least, stressor severity may be defined by the kinetics of neurotransmitter release associated with affect and motivation and putative neurotransmitters which modulate individual experiences associated with anxiety and cognitive appraisal of environmental stimuli (see Zacharko et al., 1995). The following sections detail the mesolimbic neuropeptide alterations, specifically cholecystokinin (CCK) and enkephalin (ENK), associated with mild stressor imposition and the propensity of such neural variations to influence anxiety and motivation.

Neurogenic Stressors: Brain Region Specific Anxiogenic Release

There is a considerable data base which characterizes release of mesolimbic DA in response to a variety of stressors and the potential influence of such mesolimbic DA activity on affect, motivation and anxiety (see Zacharko et al., 1995 for review). Moreover, the profile of mesolimbic neuropeptide/DA release in response to a stressor may provide a stressor severity index, associate the course or expression of behavioural change among infrahuman subjects to brain region specific neurochemical alteration and parallel symptom manifestation in human subjects. Altered CCK in specific central sites and symptom expression are intriguing in view of the plethora of data implicating CCK and anxiety induction (Bradwejn et

al., 1990; Dauge & Lena, 1998; Griebel, 1999; Zacharko et al., 1995) and perhaps depression (Lofberg et al., 1998). In rats, 60 minutes of footshock enhanced hypothalamic CCK release, while exposure of rats to 2, 4, 10 or 30 minutes of footshock increased CCK release from the prefrontal cortex (Siegel et al., 1984; Siegel et al., 1987). It should be underscored that 60 minutes of 1 mA footshock exposure is a severe stressor. Indeed, data collected in our laboratory revealed that the presentation of only two shocks within a 2 minute session (e.g., 12 seconds of footshock) is sufficient to induce anxiety in the light-dark paradigm (MacNeil et al., 1997). Severe stressors have also been associated with hippocampal release of diazepam binding inhibitor (DBI) (Alho et al., 1985; Ferrarese et al., 1991), corticotropin releasing factor (CRF) in the paraventricular nucleus of the hypothalamus, central amygdaloid nucleus and locus coeruleus (Anderson et al., 1993; Chappell et al., 1986; Hatalski et al., 1998; Helmreich et al., 1999; Imaki et al., 1991; Jezova et al., 1999; Nemeroff, 1992) and ß-carboline (e.g., ß-CCM) in the cerebral cortex (Novas et al., 1988). Among depressed subjects, a positive correlation was detected between central DBI and CRF levels (Heim & Nemeroff, 1999; Roy et al., 1989; Roy, 1991) as well as CCK and CRF availability (Geracioti et al., 1999). Owing to the conspicuous sensitivity of mesolimbic sites to relatively innocuous stressors, it appears counterintuitive to assign severity rating to peptide release per se. Perhaps norepinephrine (NE) recruitment in the hippocampus, hypothalamus and locus coeruleus (Legault et al., 2000) as well as serotonin (5-HT) in the raphe system (Bonci & Malenka, 1999; Yoshimoto & McBride, 1992) contribute to DA and CCK release in the VTA, mesocortex, nucleus accumbens and amygdala in characterizing stressor severity and defining attending dysphoric and anxiogenic symptoms.

While central NE and 5-HT alterations are ordinarily associated with severe stressors (Hwang et al., 1999; Zacharko & Anisman, 1991), relatively milder stressors promote mesolimbic DA alterations (Cabib et al., 1988; Deutch et al., 1985; Imperato et al., 1989; Keefe et al., 1990; Watanabe, 1984). Cholecystokinin is colocalized with DA in the ventral tegmental area (VTA) and mesencephalic DA/CCK neurons innervate the nucleus accumbens, amygdala and the mesocortex (mPFC) (Hokfelt et al., 1980; Hokfelt et al., 1991; Seroogy et al., 1989; Studler et al., 1981). It will be recalled that Siegel et al. (1984) reported elevated mesocortical CCK levels following 10 minutes of 1 mA footshock in rats, while 60 minutes of 1 mA footshock was necessary to induce comparable elevations of hypothalamic CCK (Siegel et al., 1987). In other laboratories, in vivo microdialysis revealed that a 2 minute intermittent exposure of rats to ether, 30 minutes of restraint or systemic yohimbine increased mesocortical CCK release (Nevo et al., 1996). Not surprisingly, diazepam pre-treatment attenuated mesocortical CCK expression prompted by ether, restraint or yohimbine administration (Nevo et al., 1996). Interestingly, in vivo microdialysis revealed that electrical stimulation of the mesocortex enhanced CCK and DA release in the nucleus accumbens among rats (You et al., 1998). Taken together, 2, 4, 6, 10, 30 and 60 minutes of footshock induce a DA and CCK release gradient from selective mesolimbic sites. Indeed, severe stressors may promote the manifestation of behavioural features characteristic of severe anxiety, while milder stressors may favor subtle variations in motivation occasioned by mesolimbic DA and CCK release profiles.

Psychogenic Stressors: Mesolimbic Cholecystokinin and Dopamine Alterations

Examination of data which describe the influence of psychogenic stressors on behavioural change and neurochemical responsivity support the argument that stressor

severity may be defined by site specific CCK release. For example, Pavlasevic et al. (1993) demonstrated that predator associated olfactory cues increased (a) CCK-4 concentrations in the olfactory bulb, frontal and central cortices, dorsal striatum, nucleus accumbens, central amygdaloid nucleus and the nucleus of the solitary tract (b) cortical DA and nucleus accumbens glutamate release and (c) striatal CCK-8S concentrations. Predator exposure was also associated with enhanced instances of freezing among female rats. Interestingly, such demonstrable inactivity may characterize anxiety profiles reminiscent of enhanced CCK-8S release in the striatum. Interestingly, Harro et al. (1996) subsequently reported that mice exposed to the auditory and olfactory cues associated with conspecific decapitation displayed increased mesocortical CCK-8S binding and increased hippocampal CCK levels. Systemically administered diazepam was ineffective in ameliorating such site specific CCK alterations. Systemic administration of the CCK_B antagonist, L-365, 260, prior to or following cat exposure, prevented expression of anxiety among rats in the elevated plus maze 1 week following the 5 minute predator exposure session (Adamec et al., 1999; Adamec et al., 1997). In summary, the long term anxiogenic effects of predator exposure among rats in the elevated plus maze, for example, are likely initiated by increased mesencephalic DA activity and such behavioural sensitization may be sustained by concurrent mesolimbic CCK_B receptor activation. However, qualitatively milder stressor experiences are characterized by more discrete mesolimbic DA and peptide alterations. For example, social isolation has been associated with anxiety in the elevated plus maze (Wright et al., 1991), CCK_B receptor up-regulation in the frontal cortex (Vasar et al., 1993), increased tyrosine hydroxylase mRNA in the VTA and decreased pro-enkephalin mRNA and met-enkephalin levels in the nucleus accumbens (Angulo et al., 1991; Carden et al., 1996). However, in adult rats, brief social

isolation (Brodin et al., 1994), like acute elevated plus maze exposure (Pratt & Brett, 1995), failed to alter basolateral amygdaloid (BLA) CCK mRNA or CCK levels, respectively. However, adult rats isolated from weaning exhibited increased CCK mRNA in the basolateral amygdala, prefrontal cortex, hippocampus and VTA relative to group housed animals (Del Bel & Guimaraes, 1997). Taken together, these data suggest that salient psychogenic stressors may elicit site-specific CCK alterations and conceivably define the perception of stressor severity.

While it has been well documented that neurogenic stressors promote anhedonia in some strains of mice as revealed by attenuated responding for previously rewarding brain stimulation from mesolimbic areas (Zacharko & Anisman, 1991; Zacharko et al., 1998; Zacharko et al., 1990), the impact of psychogenic stressors on motivation remains to be determined. Yet, physical stressors (e.g., restraint, footshock) (MacNeil et al., 1997; Nevo et al., 1996; Siegel et al., 1987) and some psychogenic stressors (e.g., predator cf. predator odor) (Blanchard et al., 1997; Pavlasevic et al., 1993) which influence central CCK provoke fear (Griebel et al., 1996) and protracted indices of anxiety (Adamec, 1997) among rats and mice. Taken together, mild stressors may elicit specific mesolimbic neurochemical alteration and provoke parallel behavioural alterations among infrahuman subjects.

Stressors and Psychological Disturbance:Organismic, Cognitive and Neurochemical Variables

It will be recalled that stressor severity operationalized along parametric lines may suggest specific central neurotransmitter alteration. Moreover, cognitive appraisal of the environmental insult and available coping strategies may influence perception of stressor controllability, the severity of the aversive encounter and ensuing behavioural responsivity among infrahuman and human subjects. It should be emphasized that cognitive and

performance linked therapeutic interventions among neurotic subjects frequently include coping strategies to reduce anxiety during pharmacotherapy (Nagy et al., 1993; Shear et al., 1991; Spiegel et al., 1994). Such interventions presumably reduce the saliency of stressor cues which precipitate anxiety. In fact, the efficacy of performance associated phobic treatment can be reliably linked to cognitive appraisal of performance self-adequacy and coping repertoires (Williams et al., 1989). It should be considered that while mild stressors or the cues associated with aversive events may elicit cognitions which re-enlist neurochemical variations associated with the original stressor experience (Ahmed & Koob, 1997; Erb et al., 1998), coping may prompt anxiolytic agent release. For example, perceived control over footshock enhances release of benzodiazepine-like agents among rats (Drugan et al., 1994, 1997; Piva et al., 1991). Yet, the source of such neurochemical activity may be associated with mesocorticolimbic sensitivity to stressor controllability. Indeed, the release of 'anxiolytic' agents following stressor imposition has been detected in the amygdala (Kang et al., 1999), the mesocortex (Cabib & Puglisi-Allegra, 1996), the VTA and the nucleus accumbens (Dziedzicka-Wasylewska & Papp, 1996). Indeed, 'anxiolytic' enkephalin release among infrahuman subjects may, in some instances, diminish the propensity of restraint, for example, to sustain protracted alterations (e.g., 1 week) of mesocortical DA release (Cuadra et al., 1999) and anxiety among animals in the light-dark paradigm (Cancela et al., 1995). Central administration of enkephalin agonists prior to or following neurogenic stressor encounter attenuated stressor associated reductions in locomotor activity (Hebb et al., 1997) and mesolimbic brain stimulation (Zacharko et al., 1998) among mice. Systemic administration of met-enkephalin prior to restraint attenuated the stressor induced increase in

plasma corticosterone among mice (Sverko et al., 1997), while chronic administration of the δ receptor antagonist, naltrindole, from birth to postnatal day 19 inhibited increased corticosterone release in 25 day old female rats to an ensuing 3 minute swim session (Fernandez et al., 1999). Moreover, systemic administration of RB-101, an enkephalinase inhibitor, attenuated while naltrexone enhanced freezing to shock associated cues in mice (Baamonde et al., 1992; Calcagnetti & Schechter, 1994). The enkephalinase inhibitor also diminished escape deficits among rats previously subjected to footshock. The trophic influence of RB-101 on stressor induced escape deficits were attenuated by prophylactic administration of the DA receptor antagonist, SCH-23390, or naltrindole, (Tejedor-Real et al., 1998). In contrast, naloxone potentiated the escape deficits induced by inescapable shock among rats (Tejedor-Real et al., 1998). Interestingly, endogenous opioids have been implicated in the therapeutic efficacy of some antidepressant agents (DeFelipe et al., 1985; 1989). Chronic imipramine treatment, for example, promoted μ-receptor expression in the hippocampus and frontal cortex (de Gandarias et al., 1998) and inhibited the enkephalin degrading aminopeptidase in rats (De Gandarias et al., 1997; Gallego et al., 1998). Taken together, coping and antidepressant agents favor mesolimbic enkephalin release among infrahuman subjects which may blunt the impact of the stressor or stressor associated cues.

The propensity of enkephalin to attenuate stressor experience among infrahuman subjects is reminiscent of CCK_B receptor antagonism in the conditioned suppression of motility (CSM) paradigm. Indeed, CCK_B agonists increase, while CCK_B antagonists decrease, immobility among shocked rats to apparatus cues previously associated with the stressor in the CSM forced swim paradigm (Derrien et al., 1994). The CSM appears responsive to

antidepressant but not anxiolytic intervention (Kameyama et al., 1982; 1985; Derrien et al., 1994). The antidepressant influence of CCK_B antagonists in the CSM, as revealed by a decrease in stressor induced motility loss, was potentiated by systemic administration of RB-101 and blocked by naltrindole administration (Hernando et al., 1996; Smadja et al., 1995). In a comparable investigation, RB-101 increased motility in response to shock associated cues in rats. The RB-101 induced motility increments were blocked by PD-134, 308, a CCK_B antagonist, microinjected into the rostral nucleus accumbens and the central amygdaloid nucleus, but not the caudate (Smadja et al., 1997). Interestingly, chronic benzodiazepine or antidepressant withdrawal elicits significant anxiety among human (Fontaine et al., 1984; Otto et al., 1993) and infrahuman subjects (Hughes et al., 1990; Singh et al., 1992). Such behavioural reactivity to anxiolytic withdrawal has been linked to increased CCK-8S receptor density in the hippocampus and frontal cortex (Harro et al., 1990) and decreased met-enkephalin immunoreactivity in the nucleus accumbens (Kurumaji et al., 1988; Przewlocki et al., 1997). It should be considered that CCK_B receptor antagonism and/or δ-receptor activation facilitates mesolimbic DA release and modulates behavioural reactivity during antidepressant interventions.

Interestingly, anatomical studies reveal that CCK and enkephalins have comparable distributions within numerous mesolimbic sites associated with mood as well as learning and memory (Cooper, 1991; Gall et al., 1987, Mansour et al., 1988; Pohl et al., 1990). The colocalization of neuropeptides within mesolimbic sites typically associated with reward or arousal suggests that alterations in motivation or anxiety may not be mutually exclusive. Indeed, systemic administration of the opioid antagonists, naloxone and naltrexone, enhanced

cardiovascular indices induced by a cognitive stressor among normal and cardiac patients (Fontana et al., 1997; 1998; McCubbin et al., 1998). Interestingly, naloxone and naltrexone also decreased approach behaviour among phobic individuals (Arntz, 1993; Janssen & Arntz, 1996; 1997) and attenuated the anxiolytic influence of diazepam among patients awaiting surgery (Duka et al., 1982). A relationship between increased endogenous opioids and coping has been suggested pertaining to human and infrahuman subjects (Goodwin & Barr, 1997; Jamner & Leigh, 1999) although the precise nature of such a relationship remains to be defined. Interestingly, naloxone and the mixed $CCK_{A/B}$ antagonist, proglumide, eliminate placebo induced analgesia among post-operative pain patients (Benedetti, 1996; Benedetti et al., 1997; Benedetti & Amanzio, 1997), suggestive of a CCK/opioid influence on anticipatory anxiety. The demonstration that anticipation of stressor encounter influences CCK activity in humans (Harro et al., 1992; Phillipp et al., 1992) and animals (Becker et al., 2001) is consistent with such an interpretation. In rats, chronic diazepam or flurazepam treatment reduced the excitatory effect of CCK-8S on CA3 hippocampal neurons in vitro suggesting a putative CCK link to the anxiolytic action of some benzodiazepines (Bouthillier & DeMontigny, 1988). Moreover, naloxone attenuated the anxiolytic properties of diazepam and chlordiazepoxide in mice in the light-dark and elevated plus maze paradigms (Agmo et al., 1995; Belzung & Agmo, 1997). Naloxone also potentiated the anxiogenic effects of sub-threshold doses of CCK-8S and CCK-4 in the elevated plus maze in rats (Koks et al., 1998). Among non-human primates, naloxone increased and morphine decreased vocalizations among infants separated from their mother (Kalin et al., 1988). These findings were replicated in rats exposed to a predator. In particular, morphine decreased (Shepherd et al., 1992) while naloxone increased (Blanchard et al., 1991) ultrasonic emissions among rats exposed to a cat.

Taken together, acute exposure of infrahuman subjects, non-human primates and humans to neurogenic or psychogenic stressors accelerates DA turnover, promotes enkephalin release and CCK availability in the mesocortex, nucleus accumbens and VTA. Such a neurochemical signature within the mesolimbic system may be influenced by the severity of the environmental experience and determine behavioural responsivity (Giardino et al., 1999; Imperato et al., 1992; Izumi, 1998; Siegel et al., 1984). In effect, these data suggest that activation of CCK neurons within mesolimbic sites and the recruitment of hypothalamic and non-DA CCK neural populations may be indicative of increasing stressor severity. Nevertheless, release of endogenous enkephalins may attenuate CCK availability and limit the stressor experience.

Examination of clinical data suggests that the nature and severity of stressful life experiences influence the nature and persistence of psychological disturbance. For example, major life events, including death or chronic illness of a loved one, are likely to provoke a depressive episode among female subjects (Bifulco et al., 1998; Steiner, 1992). In addition, coping influences depression severity. Finally, significant life events which involved threat of physical danger are more likely to sustain anxiety and exacerbate depressive symptoms (Brown, 1993; Faravelli et al., 1985; Finlay-Jones & Brown, 1981; Garnefski et al., 1990; Manfro et al., 1996; Roy-Byrne et al., 1986) in young male subjects (Garnefski & Diekstra, 1997). However, it should be underscored that comorbid symptoms of anxiety and depression often appear among individuals who have sustained chronic life stressors and have previously been exposed to major trauma prior to age 19. Indeed, major life stressors in females prior to age 19 have been associated with ensuing prevalent comorbid psychological disturbance (Garnefski et al., 1990; Kivela et al., 1998; Mancini et al., 1995; Manfro et al.,

1996; Weiss et al., 1999). Patients with anxiety and depressive comorbidity are typically more anxious, fearful of criticism, unassertive, socially impaired (Roy-Byrne et al., 1992) and more likely to relapse following termination of pharmacotherapy relative to non depressed anxious patients (Gaynes et al., 1999; Goldberg, 1999). It may be relevant that major affective disorder with prominent neurotic comorbidity has often led to the induction of depression with comorbid expression of panic (Brown et al., 1996; Keller & Hanks, 1993; Parker et al., 1999). In effect, anhedonia and anxiety may undergo concurrent exacerbation. Perhaps early life experiences entrain cognitive sets or induce rumination conducive to the maintenance of neurochemical variations which sustain psychological disturbance. Interestingly, arguments for behavioural and neurochemical conditioning and sensitization have been proposed to account for clinical relapse, relapse chronicity and protracted symptom exacerbation (Dykman et al., 1997; Kessing et al., 1998; Post et al., 1986; Post, 1992; Post & Weiss, 1998; Roy-Byrne et al., 1985; Segal et al., 1996; Zacharko et al., 1995).

It should be underscored that the prevalence of familial illness, the perception of such events and the impact of sick role behaviour cannot be dismissed. Indeed, it has been posited that such factors contribute to the onset and the course of psychological disturbance (Ahmad et al., 1992; Landerman et al., 1991; Pollack et al., 1996; Rosenbaum et al., 1988; Roth, 1996; Shear, 1996; Van Os & Jones, 1999; Whitehead et al., 1994). For example, some laboratories have argued for a relationship between separation anxiety, school phobia and familial illness, and the development of severe anxiety among children in early adulthood (Bakish, 1994; Brown et al., 1999; Ford & Kidd, 1998; Free et al., 1993; Heim & Nemeroff, 1999; Klein et al., 1995; Pollack et al., 1996; Shear, 1996). To be sure, premorbid reactivity to situational variables has been offered as a risk factor in anxiety (Kagan et al., 1988; Whitehead et al.,

1994). Indeed, adolescents with a parental history of anxiety exhibited enhanced startle to air puff threat (Grillon et al., 1998). Likewise, panic patients with a history of early life stressors (e.g., childhood separation, familial panic indices, agoraphobia) exhibit a more rapid age of panic onset relative to individuals who fail to report such events (Battaglia et al., 1995). In this respect, panic severity and illness duration are clearly evident. Such an analysis favors assessment of the cognitive repercussions associated with anxiety associated disorders and individual perception of stressor saliency. It should be noted parenthetically that non-human primates reared under stressful conditions (e.g., mothers confronted with variable foraging demands) reveal aberrant behaviour (e.g., hyperactivity, clinging, behavioural inhibition) (Rosenblum & Paully, 1984) and exhibit altered central DA concentrations relative to maternally fostered animals. The former stressor profile may impede subsequent ability of the infant to cope with life stressors (Coplan et al., 1996; Coplan et al., 1998). Exposure of pregnant dams to restraint increased DA binding in the nucleus accumbens and promoted locomotor activity to challenge doses of d-amphetamine in adult offspring (Henry et al., 1995). Moreover, prenatally stressed rats demonstrated demonstrable anxiety in response to novelty (e.g., open field, Y-maze, elevated plus maze) relative to rats which were not exposed to the stressor (Vallee et al., 1997). In a similar vein, in vivo microdialysis revealed that pups isolated briefly from the nest and the dam (e.g., 5 minutes) displayed enhanced accumbens DA turnover when these animals matured relative to rats which were not isolated as pups (Kehoe et al., 1996). To date, evidence for an enduring influence of site specific central CCK alterations among human or non-human primates exposed to early life stressors are unavailable. However, central CCK concentrations have been linked to the severity of neurotic forms of depression. In particular, depressed individuals with a suicidal history

displayed elevated cerebrospinal (CSF) CCK levels relative to individuals with milder forms of depression (Lofberg et al., 1998). Although suicidal ideation and intent have been linked to central 5-HT perturbations (Pandey, 1997; Roy, 1999; Roy & Pollack, 1994; Verkes et al., 1998), the prevalence of CCK/5-HT interactions (Bloom & Morales, 1998; Rex & Fink, 1998; Raiteri et al., 1993; To & Bagdy, 1999) in mesolimbic sites associated with mood and anxiety suggests that CCK may sustain behavioural pathology. In summary, early negative life experience among human and infrahuman subjects promote mesolimbic CCK/DA alterations, enhance vulnerability to behavioural disturbance and influence the profile of symptom manifestation to ensuing stressor encounter.

Conditioning and Sensitization of Mesolimbic Dopamine, Cholecystokinin and Enkephalin in the Development and Exacerbation of Psychological Disturbance

Behavioural sensitization refers to an augmentation of behaviour among animals challenged with a low psychostimulant dose (Ohmori et al., 1995; Kuribara, 1996) or brief footshock (Robinson et al., 1987) following acute or chronic d-amphetamine treatment and/or acute or chronic neurogenic stressor exposure. Behavioural change among animals exposed to footshock, d-amphetamine or cocaine may appear within 24 hours and may persist for one year following initial stressor presentation and ensuing psychostimulant or stressor re-exposure (Antelman et al., 1980; Jackson & Nutt, 1993; Paulson et al., 1991; Post et al., 1992; Robinson & Becker, 1986; Robinson, 1988; Steketee et al, 1992; Sorg & Kalivas, 1991). Likewise, stereotypy and locomotor activity may be augmented within 24 hours as well as one month following acute or chronic maintenance doses of d-amphetamine or cocaine (Battisti et al, 1999; Robinson et al, 1982). Among human subjects, chronic d-amphetamine (0.25 mg/kg x 3/day) increased subjective and experimenter ratings of activity, mood and

speech production by the third self-administration session (Strakowski et al. 2001; Strakowski et al, 1996; Strakowski & Sax, 1998). Taken together, augmented behavioural reactivity among human and infrahuman subjects is evident at abbreviated or protracted intervals following initial pharmacological or stressor exposure and ensuing pharmacological or stressor challenge (see Paulson et al, 1991; Paulson & Robinson, 1995).

The development and expression of psychomotor induced behavioural sensitization is also influenced by contextual cues previously associated with psychostimulant or stressor application. For example, sensitization to the locomotor inducing influence of cocaine (e.g., 10 mg/kg) 24 hours following acute cocaine administration (e.g., 40 mg/kg) was only evident in the identical environment in which mice initially received cocaine (Jackson & Nutt, 1993). In a similar vein, sensitization of stereotypy to d-amphetamine (7 mg/kg) 48 hours following acute amphetamine pre-treatment (14 mg/kg) was also dependent upon environmental cues previously associated with d-amphetamine administration in mice (Battisti et al, 1999). Interestingly, acute d-amphetamine (1 mg/kg) administered immediately following a protected exposure of a rat to a cat increased locomotor activity only in the presence of cat litter 30 minutes following psychostimulant administration (Williams & Barber, 1990). Finally, home or novel cage chronic cocaine administration (10 mg/kg x 10 days) in rats increased activity on days 4, 7 and 10 in comparison to day 1 of the chronic drug schedule or the behaviour of saline treated animals. Chronic cocaine treated mice displayed prominent stereotypy 72 hours following acute cocaine challenge relative to 24 hour challenge. Augmented stereotypy and locomotor activity were only evident when mice were challenged in the environment defining the initial cocaine experience (Post et al, 1981).

Commensurate with the demonstration that development and expression of psychomotor induced behavioural sensitization is influenced by environmental cues, rats "freeze" immediately following introduction to the environment previously (i.e., within 24 hours) associated with footshock (Blanchard & Blanchard, 1969; Valentinuzzi et al, 1998; Wallace & Rosen, 2001). It should be noted that acute footshock enhanced blood pressure in rats to a shock prod 14 days following initial stressor exposure relative to non shocked animals (Bruijnzeel et al, 2001). Interestingly, restraint imposed on mice immediately, but not 30 minutes, following chronic meth-amphetamine (2 mg/kg) or cocaine (20 mg/kg) treatment (7 times at 3 day intervals) inhibited locomotor activity to psychostimulant treatment 72 hours following the final drug-restraint session (Kuribara, 1996; Kuribara, 1998). It should be underscored that behavioural sensitization may also define reduced responsivity among animals exposed to a chronic stressor or to the cues previously associated with the stressor. For example, it has been demonstrated in this laboratory that a 6 second exposure to footshock decreased locomotor activity among mice previously exposed to 60 minutes of the identical stressor 18 days earlier (Hebb et al., 1997). Taken together, psychostimulants (e.g., amphetamine, cocaine), neurogenic (e.g., footshock, restraint) and some psychogenic (e.g., predator, predator odor) stressors offer inter-changeable stimulus configurations conducive to the induction of behavioural sensitization among animals. Moreover, a role of environmental conditioning factors, including the initial expression of a drug-induced or stressor response, in the development and persistence of behavioural sensitization is in evidence. In effect, the application of a conditioning/sensitization model following stressor imposition among human subjects may define situational variables and provide a putative framework concerning the saliency and time course of environmental events.

Behavioural indices of sensitization may be associated with enhanced sensitivity of some mesolimbic DA sites. Indeed, such sensitivity has been linked to the VTA (Kalivas, 1985), the shell of the nucleus accumbens (Parkinson et al., 1999), the central and basolateral amygdaloid nuclei (Goosens & Maren, 2001; Shors et al., 1997; Shors & Mathew 1998; Wallace & Rosen, 2001) and the mesocortex (Karler et al., 1998; Sorg & Steketee, 1992). It should be underscored that identification of mesolimbic sub-nuclei sites (e.g., shell cf. nucleus accumbens core) underlying behavioural sensitization is limited. In retrospect, chronic psychostimulant administration increases extracellular DA in the nucleus accumbens and VTA and concentrations of DA induced by acute d-amphetamine (i.e., 2 mg/kg) and cocaine (i.e., 15 mg/kg) are enhanced in animals following acute or chronic footshock (i.e., .45 mA) (Hamamura et al, 1997; Kalivas & Duffy, 1993; Post et al., 1992). Such neurochemical indices reflect acute psychostimulant or stressor challenge following (e.g., within 24 hours) acute or chronic (e.g., 14 days) footshock or psychostimulant administration (Antelman et al., 1980; Davis, 1989; Hamamura & Fibiger, 1993; Imperato et al., 1996; Jackson & Nutt, 1993; Kalivas & Duffy, 1993; Paulson et al., 1991; Post et al., 1992; Prasad et al., 1995; Robinson & Becker, 1986; Robinson et al., 1987; Robinson et al., 1988; Sorg & Kalivas, 1991). Rats treated with d-amphetamine (4 mg/kg) for 14 days exhibited increased DA metabolism in the prefrontal cortex in response to acute footshock (i.e., 0.4 mA) 7 days following d-amphetamine relative to non d-amphetamine treated animals (Hamamura & Fibiger, 1993). Acute d-amphetamine (1.25 mg/kg) enhanced DA release from the rat nucleus accumbens to a subsequent injection of d-amphetamine (1.25 mg/kg) administered 3-5 weeks later (Robinson et al, 1982). Chronic footshock in rats (i.e., 2 mA x 2/day, 5 days) progressively increased DA turnover in the nucleus accumbens and frontal cortex as revealed

by 3-MT concentrations on Day 10 compared to Day 1. Rats which were re-exposed to footshock 14 days following chronic footshock demonstrated enhanced mesocortical DOPAC, HVA and 3-MT levels and attenuated DOPAC concentrations in the nucleus accumbens relative to non-shocked animals (Chrapusta et al, 1997). Indeed, mere exposure of rats to the shock apparatus previously associated with footshock increased HVA levels in the medial prefrontal cortex relative to non shocked animals (Tsuchiya et al, 1996).

It should be underscored that DA in the VTA may initiate while nucleus accumbens and amygdaloid DA release may sustain behavioural expression of sensitization associated with a stressor (e.g., Hamamura & Fibiger, 1993; Eichler & Antelman, 1979; Kalivas et al., 1988; Kalivas et al, 1993; Kalivas & Stewart, 1991; Wallace & Rosen, 2001). In effect, behavioural sensitization is initiated by transient mesolimbic neurochemical events which alter organismic processing of environmental and pharmacologically relevant stimuli. For example, intra-VTA administration of SCH-23390 (0.5-1.0 μg) attenuated the acute locomotor effects of amphetamine and blocked expression of sensitization to acute amphetamine (0.5 mg/kg) following a chronic d-amphetamine regimen (Stewart & Vezina, 1989). Likewise, augmented locomotor activity and nucleus accumbens DA availability following acute intra-VTA (2.5 μg) d-amphetamine 3 weeks following a chronic d-amphetamine schedule (2.5 μg every 3- 4 days x 12 days) among rats was blocked when chronic d-amphetamine treatment was accompanied by systemic co-administration of a DA antagonist (Vezina & Stewart, 1989; Vezina, 1993; Vezina, 1996).

The expression of sensitization has been defined as an enduring mesolimbic change mediating prolonged behavioural alteration following chronic drug or stressor treatment

(Kalivas & Duffy, 1993). For example, re-exposure of animals to a mild stressor or a previously neutral psychostimulant dose following chronic amphetamine treatment increases nucleus accumbens activity, extracellular DA availability and locomotor activity (Antelman et al., 1980; Cassens et al., 1980; Pierce & Kalivas, 1995). Moreover, in vivo microdialysis revealed that rats and mice challenged with a low d-amphetamine dose (e.g., .05 mg/kg) 14 days following chronic d-amphetamine (1 mg/kg x 14 days) demonstrated enhanced locomotor activity and increased amygdaloid and nucleus accumbens DA activity relative to saline treated subjects (Harmer et al., 1997; Harmer & Phillips, 1999; Vezina, 1996). The increase in DA in the amygdala and nucleus accumbens following chronic amphetamine treatment was paralleled by decreased DA activity in the mesocortex (Karler et al., 1998). Interestingly, one week following a 7 day d-amphetamine (2 mg/kg) schedule, rats demonstrated enhanced extracellular amygdaloid DA to a neutral stimulus (i.e., tone, light) previously paired with sucrose pellet delivery. Arguments for a link between amygdaloid DA activity and the facilitation of associative learning was offered in view of the demonstration that rats exhibited enhanced acquisition of the conditioned response (i.e., sucrose pellet retrieval) (Harmer & Phillips, 1998 and 1999). Rats exposed to a novel environment previously associated with footshock displayed conspicuous freezing as well as increased DA activation (e.g., c-fos) within the lateral amygdala (Rosen et al, 1998). Moreover, mesoamygdaloid DA inhibition effected by intra-lateral amygdaloid SCH 23390 administration or intra-VTA quinpirole administration attenuated freezing to the conditioned environment (Nader & LeDoux, 1999). Taken together, alterations of VTA DA activity may contribute to the initiation of sensitized behavioural responses, while the nucleus accumbens and amygdala DA activity may promote expression of behavioural sensitization induced by

psychostimulant and stressor treatment. In particular, the nucleus accumbens may integrate environmental cues encoded by the mesencephalon, the amygdala and mesocortex in response to stressors or pharmacological manipulations which mimic stressor effects. Such neural integration is presumed to initiate and sustain behaviour (Ikemoto & Panksepp, 1999; Ladurelle et al., 1994; Mogenson et al., 1980, 1985; Mogenson & Nielsen, 1984; Mogenson & Yang, 1991; Pert et al., 1990; Phillips et al., 1993; van den Bos et al., 1991; Vezina & Kim, 1999; Yim & Mogenson, 1982).

There are sufficient data to suggest that neurotransmitters and neuropeptides colocalized with mesolimbic DA influence behavioural sensitization. Indeed, enkephalin and CCK release within the nucleus accumbens, VTA, amygdala and mesocortex influence mesolimbic DA and contribute to the behavioural expression of sensitization. For example, chronic psychostimulant administration in rats is associated with increased accumbal prepro-enkephalin mRNA (Zhang et al., 1997) and DA increases in the nucleus accumbens shell but not the core (Heidbreder & Feldon, 1998; Heidbreder et al., 1999). Increased locomotor activity following acute footshock or d-amphetamine (1.0 mg/kg) in rats, 48 hours following chronic intra-VTA administration of D-Ala^2, Met^5-enkephalinamide (DALA) (1 μg) increases DA turnover in the mesocortex, nucleus accumbens and mesencephalon (Kalivas et al., 1986; Kalivas & Stewart, 1991; Robinson et al., 1987). Interestingly, increased DA turnover (i.e., DOPAC) in the mesocortex and nucleus accumbens and elevated locomotor activity have been also linked to μ receptor activation in the VTA (Kalivas & Abhold, 1987; Vezina et al., 1987). For example, chronic intermittent intra-VTA administration of d-amphetamine (2.5 μg) enhanced locomotor activity to an acute systemic injection of the μ agonist, morphine, 3

weeks following chronic d-amphetamine relative to saline treated rats. Such a behavioural effect among rats was not evident when d-amphetamine was chronically infused into the nucleus accumbens (Vezina & Stewart, 1990). Acute injection of DALA into the VTA dose dependently increased the DOPAC/DA ratio in the nucleus accumbens (Churchill & Kalivas, 1992; Kalivas & Duffy, 1990) and enhanced locomotor activity to a challenge dose of d-amphetamine (1 mg/kg) relative to saline treated animals. Likewise, mild footshock (e.g., 0.14 mA) in rats one week following intra-mesencephalic DALA administration effected a comparable behavioural change (Kalivas, 1985; Kalivas et al., 1986). Finally, administration of the μ antagonist, β-funaltrexamine, but not naltrindole, attenuated d-amphetamine induced DA increase from the rat nucleus accumbens (Schad et al., 1996). Other investigations have revealed that in addition to μ receptor activation in the VTA, μ and δ receptor agonists administered into the nucleus accumbens enhance locomotor activity following stressors or pharmacological manipulations which mimic stressors (Svingos et al., 1999).

It should be underscored that while μ and δ agonists contribute to the induction of behavioural sensitization, other neurotransmitter systems likely contribute to the maintenance of protracted behavioural change. For example, repeated intraventricular administration of the selective μ agonist, D-Ala^2,MetPhe^4,Gly-ol^5-enkephalin (DAMGO) (i.e., 0.003-0.01 μg) preceding chronic intermittent meth-amphetamine (2 mg/kg) administration, attenuated the progressive increase in locomotor sensitization. The increase in locomotor activity was conspicuous following the third administration (i.e., Day 10) of the psychostimulant relative to Day 1 (Toyoshi et al., 1996). Moreover, co-administration of naltrindole (0.1-3.0 mg/kg) and cocaine (20 mg/kg, 3 days) blocked locomotor activity in rats

to acute cocaine (20 mg/kg) 48 hours following chronic cocaine-δ antagonist co-administration schedule (Heidbreder et al., 1993). In contrast, the identical dose of naltrindole administered 15 minutes prior to cocaine challenge (20 mg/kg) failed to attenuate the increased locomotor response of rats (Heidbreder et al., 1996). Taken together, these data suggest that μ and δ receptors participate in the development while the maintenance of behavioural sensitization is maintained by enkephalin and CCK colocalized with mesolimbic DA. Clearly the distribution and putative interactions between CCK and mesolimbic enkephalin (e.g., VTA, nucleus accumbens, amygdala) contribute to the emergence of behavioural sensitization. Yet, the nature of the relationship between CCK, enkephalin and mesolimbic DA to the immediate and long-term behavioural consequences of stressor encounters remain to be defined.

In retrospect, chronic meth-amphetamine administration in rats (i.e., 3-6 mg/kg x 14 days) decreased CCK concentration, CCK mRNA expression and CCK binding in the mesocortex (Bing et al., 1997; Fukamauchi, 1996; Kawai et al., 1997; Suzuki & Morijo, 1989; Suzuki et al., 1993; Vecchiola et al., 1999; Yoshikawa et al., 1994), VTA, as well as the medial and caudal aspects of the nucleus accumbens (Hurd et al., 1992). In rats, ventricular or nucleus accumbens administration of the CCK_A/CCK_B agonists CCK-8S (Weiss et al., 1988) or ceruletide (Kihara et al., 1993; Kuribara, 1995) antagonized the locomotor activating effects of low dose d-amphetamine. Systemic administration of L-365,260 (0.01 or 1.0 mg/kg) but not the CCK_A antagonist devazepide (0, 0.001-1.0 mg/kg) followed by d-amphetamine (1.5 mg/kg) for 1 week attenuated the enhanced locomotor response to acute d-amphetamine (0.75 mg/kg) 10 days following chronic amphetamine in rats. In contrast, administration of devazepide but not L-365, 260 attenuated the sensitized locomotor

response to d-amphetamine administered 30 minutes prior to acute d-amphetamine (0.75 mg/kg) challenge 10 days following chronic d-amphetamine administration in rats (Wunderlich et al., 1997). The results of this experiment suggested that CCK_B receptor activation in the nucleus accumbens and mesocortex attenuates the initiation of sensitization induced by the nucleus accumbens. In contrast, CCK_A receptor activation attenuates the expression of behavioural sensitization to d-amphetamine.

The shell and the core of the nucleus accumbens receive prominent DA-CCK colocalized fibers from the VTA and substantia nigra (Lanca et al., 1998), respectively which may define specific behavioural alterations (e.g., the promotion of locomotor activity cf. freezing) following stressor imposition. In addition to a shell-core consideration of the nucleus accumbens a rostral-caudal division also provides conspicuous discrepancies in CCK availability. For example, an increase in nucleus accumbens CCK activity can potentiate or attenuate d-amphetamine induced increases in locomotor activity. In particular, CCK-8S microinjection into the caudal but not the rostral nucleus accumbens effects a DA agonist-like influence on d-amphetamine-induced locomotor activity (Vaccarino & Rankin, 1989). Rostral-caudal variability in the distribution and density of CCK_A and CCK_B receptors in the nucleus accumbens allows CCK-8S to potentiate or inhibit behavioural change among stressor or amphetamine sensitized animals. Cholecystokinin-8S in the rat posteromedial nucleus accumbens, with a prominent CCK_A receptor distribution, was associated with reduced open arm entries in the elevated plus maze which was reversed by the CCK_A antagonist L364,716. In rats, administration of the CCK_A antagonist, devazepide blocked the augmented locomotor response to an acute dose of psychostimulant immediately (Vasar et al., 1991 c.f. Kobayahi et al., 1996) and to a psychostimulant challenge dose 10 days following chronic d-

amphetamine treatment (Desousa et al., 1999). In contrast, administration of the CCK_B antagonist, L365-260, potentiated d-amphetamine induced hyperactivity (Higgins et al., 1994). Intra-accumbens CCK-8S administration potentiated d-amphetamine enhanced (1.0 mg/kg) locomotor activity in rats while simultaneous intra-accumbens injection of enkephalin attenuated the locomotor activity promoted by CCK-8S and d-amphetamine co-administration (Mueller & Whiteside, 1990). These data suggest that mesolimbic CCK may be involved in the maintenance and enkephalin in the attenuation of behavioural changes associated with chronic amphetamine administration. It should be emphasized that although the current analysis is restricted to a discussion of the contribution of enkephalin, CCK and DA in effecting behavioural sensitization, there is considerable evidence to implicate glutamate, NMDA receptor activation and long-term potentiation in the protracted behavioural and neurochemical change following stressor imposition. Indeed, various laboratories have argued that long term potentiation (LTP), conducive to memory and learning, contributes to the expression of behavioural sensitization. In this respect, enhanced sensitivity to previously neutral stimuli and NMDA receptor activation of the VTA may contribute to heightened neuronal sensitivity and behavioural activation (see Bandopadhyay & Belleroche, 1999; Bonci & Malenka, 1999; Cador et al., 1999; Churchill et al., 1999; Giorgetti et al, 2001; Overton et al., 1999; Vezina & Kim, 1999; Vezina & Queen, 2000; Wolf, 1998; Wolf & Xue, 1999; Wu et al., 1993; You et al, 1997; You et al., 1998).

Data collected in this laboratory revealed that brief footshock or restraint reliably induce anxiety among infrahuman subjects in the light-dark box. For instance, decreased light chamber occupancy and inter-chamber transitions are exaggerated following central CCK-8S administration one week following drug administration relative to vehicle treated mice

(MacNeil et al., 1997). Prior stressor history of mice also influences locomotor activity to subsequent administration of enkephalin agonists (Calenco-Choukroun et al., 1991). For example, intra-VTA administration of some δ receptor agonists (e.g., DTLET, DSTBULET, BUBU) in the rat induced hyperactivity in a familiar (i.e., home cage), unfamiliar (four-hole box) and an open-field, while DAGO enhanced locomotion in the familiar but not in the unfamiliar environment or the open-field paradigm 24 hours following a 3 day habituation baseline schedule (Calenco-Choukroun et al., 1991). It may be relevant to note that the mere exposure of mice to an open field induces anxiety (e.g., immobility) (Tassin et al, 1980). Administration of CCK-8S into the posterior nucleus accumbens of rats in a novel environment (i.e., four-hole box) promoted hypoexploration and increased DA turnover, relative to rats administered CCK-8S in a familiar homecage environment which failed to display behavioural or neurochemical change. Moreover, intra-accumbal administration of CCK was only anxiogenic in an elevated plus maze among rats previously exposed to the four-hole box novel environment immediately prior to CCK administration (Ladurelle et al., 1995). In effect, prior stressor history, μ, δ and CCK activation, or neuropeptide and stressor cross-sensitization may be dependent on the nature and the contextual cues associated with anxiogenic challenge. Moreover, such stressor scenarios may define the profile of symptoms and determine vulnerability (e.g., latency to the emergence of psychological disturbance) to anxiety. Such predisposing neurochemical variations may (a) identify region specific neural sequelae contributing to behavioural sensitization and conditioning among infrahuman subjects (b) provide a parallel for the development of psychological disturbance in human subjects and (c) suggest therapeutic intervention

strategies. In effect, pharmacological interventions, which ameliorate psychological pathology, may attenuate the neuronal processes that ordinarily underlie sensitization.

Among infrahuman subjects, a variety of neurogenic (e.g., footshock, restraint) (Davis, 1990), psychogenic (e.g., novelty, predator odors) (Antelman et al., 1992; Blanchard et al., 1990; 1998 Hotsenpiller & Williams, 1997; Kavaliers et al., 1997; Kavaliers & Choleris, 1997; Zalaquett & Thiessen, 1991) and pharmacological (e.g., CCK administration) (MacNeil et al., 1997; Rex et al., 1994) stressors have been employed to simulate diverse symptom profiles associated with human pathology. Neurogenic stressors provoke reliable behavioural and neurochemical indices of sensitization among animals following acute (Chung et al., 2000; Diaz-Otanez et al., 1997; Van Dijken et al., 1992) or chronic administration schedules (Antelman et al., 1980; Cuadra et al., 1999). In addition, there are data to suggest that psychogenic stressors may induce relatively protracted neurochemical and behavioural alterations in animal models of anxiety (Adamec, 1997). Consistent with the evidence gleaned from paradigms which employ neurogenic stressors to assess anxiety, the intensity and duration of a psychogenic stressor may influence the pattern of central neurochemical and behavioural change in animal models of anxiety (Inoue et al., 1994; Marti & Armario, 1998; Zelena et al., 1999). Infrahuman investigations suggest that sex (Farabollini et al., 1996; Fernandez et al., 1999; Heinsbroek et al., 1988; Klein et al., 1998; Micevych et al., 1997; Wigger & Neumann, 1999; Wilson & Biscardi, 1994), age (Barili et al., 1998; Brodish & Odio, 1989; Harro & Oreland, 1992; Issa et al., 1990; Mabry et al., 1995; van Oers et al., 1998; Vazquez, 1998), genotype (Cabib et al., 1998; Haeri et al., 1996; Harris & Nestler, 1996; Ramos et al., 1997; Rex et al., 1996) and prior stressor history (Brier, 1989; Haeri et al., 1996; Husaini, 1997; Kashini et al., 1999; Messenger & Shean, 1998; Pohorecky et al., 1999)

influence the behavioural manifestations associated with subsequent stressor application and concomitant mesolimbic DA, CCK and opioid availability underlying alterations in anxiety and motivation. The ensuing sections of this dissertation detail the a) distribution and functional interaction of enkephalin and CCK receptor subtypes within discrete areas of the mesolimbic system associated with memory, learning, motivation and anxiety and b) data pertaining to a μ-δ/CCK interactions within the nucleus accumbens and VTA which encode the saliency of aversive experiences in animals.

The Distribution, Function and Interaction of Mesolimbic μ, δ, and CCK Receptors

Radioligand-binding and pharmacological studies have identified diverse opioid receptors and specific subtypes. For example, mesocorticolimbic and nigrostriatal μ (μ_1, μ_2), and δ (δ_1 δ_2) receptors display differential endorphin and enkephalin sensitivity (Bouret et al., 1999; Jiang et al., 1991; Mansour et al., 1996; Negri et al., 1991; Reisine, 1995; Schulz et al., 1998; Svingos et al., 1999; Wollemann et al., 1993; Zastawny et al., 1994). Immunofluorescence and ligand binding have verified enkephalin and DA colocalization in the VTA, striatum, substantia nigra, mesocortex, amygdala and the nucleus accumbens (Curran & Watson, 1995; Fallon & Leslie, 1986; Mansour et al., 1988). The neural interface of such neurotransmitters permits integration of motivation and motoric output. For example, intra-accumbal injection of morphine and DAMGO increase locomotor activity and exploration among rats in the elevated plus maze as revealed by increased open arm entries and rearing, while naltrexone and 6-OHDA ablation of the VTA attenuate such behavioural effects (Anseloni et al., 1999; Churchill et al., 1998). Indeed, there is sufficient evidence to suggest that μ_1 and δ_1 receptors in the nucleus accumbens and VTA participate in mediating

motivation (Piepponen et al., 1999; Stolerman, 1985; Wise, 1989; Wolozin & Pasternak, 1981).

Enkephalin containing varicosities, as well as μ and δ receptor subtypes, have been detected in sub-areas of the VTA (Dilts & Kalivas, 1989; 1990; German et al., 1993) and the shell of the nucleus accumbens (Svingos et al., 1999; Voorn & Docter, 1992). In particular, μ_1 and δ_1 receptor activation is associated with increased DA release in the nucleus accumbens (Churchill et al., 1995; Suzuki et al., 1997). It will be recalled that CCK_A receptor activation also increased DA activity while CCK_B receptor activation decreased DA activity in the rat nucleus accumbens (Marshall et al., 1991). Enkephalin appears to be a competitive CCK_B receptor antagonist (Murphy et al., 1992). Similarly, the non-selective CCK antagonists, proglumide and lorglumide, compete for μ and δ binding sites (Gaudreau et al., 1990). Binding of CCK-8S reduces the density and affinity of cortical δ (Johnson et al., 1987) as well as μ receptors in whole rat brain as measured in vitro (Wang & Han, 1990; Zhang et al., 1993). Moreover, the comparable endogenous ligands for the δ-opioid (i.e. enkephalin) and the CCK_B receptor (i.e., CCK-8S, CCK-4) provide competitive agonist properties (Rezvani et al., 1987). Interestingly, it has been demonstrated in vitro that neuropeptidase degradation of CCK-8S and CCK-4 competes with enzymatic degradation of leu-enkephalin (Deschodt-Lanckman & Bui, 1981; Rose et al., 1995, 1996). Taken together, μ_1 and δ_1 receptor activation attenuate CCK_B mediated DA reductions in the nucleus accumbens and VTA and may attenuate alterations in motivation and anxiety associated with a stressor.

The enkephalins and endorphins are derived from independent precursor polypeptides designated pro-enkephalin and pro-opiomelanocortin (POMC), respectively. Pro-enkephalin is conspicuous within the mesocorticolimbic system (Hurd et al., 1996), while pro-opiomelanocortin appears primarily along the hypothalamic-pituitary-adrenal axis and is the polypeptide precursor of adrenocorticotropin hormone (ACTH) (Blasquez et al., 1994). It might be noted at this juncture that chronic and relatively severe anxiety disorders are characterized by elevated CSF β-endorphin levels (Baker et al., 1997; Darko et al., 1992; Eriksson et al., 1989; Goodwin et al., 1993; Westrin et al., 1999). The expression of pro-enkephalin as well as immediate early gene activation (e.g., c-fos) have been employed to characterize neuronal responsivity to stressors and pharmacological manipulations which mimic stressors (Duncan et al., 1996; nucleus (Kollack-Walker et al., 1997; Larsen & Mau, 1994). For example, social isolation (e.g., 7 days) decreased pro-enkephalin mRNA in the nucleus accumbens in rats (Angula et al., 1991). Moreover, pro-enkephalin deficient mice display exaggerated anxiety in an open field or the elevated plus maze and increased conspecific aggression (Kieffer, 1999; Konig et al., 1996). In contrast, it has been demonstrated that increased amygdaloid pro-enkephalin expression, accomplished by amygdaloid administration of a replication-defective herpes virus carrying human prepro-enkephalin cDNA enhanced the anxiolytic properties of diazepam in the elevated plus maze in rats (Kang et al., 1999; 2000). Taken together, increased availability of pro-enkephalin and/or μ_1 and δ_1 receptor activation in the amygdala and nucleus accumbens is associated with an attenuation of anxiety. Moreover, the proximal neuroanatomical distribution of CCK and enkephalin in the VTA, nucleus accumbens and amygdala promoting learning/memory, and

motivation suggests a μ-δ/CCK link in the modulation and expression of anxiety or stressor-related behaviours.

Consideration of μ-δ/CCK_B Interaction and Encoding of Salient Environmental Experiences

The anxiety associated with systemic CCK-4 administration and central CCK_B receptor activation among infrahuman and panic among human subjects are well documented (see van Megen et al., 1996 for review). Two CCK_B receptor configurations appear to contribute to the expression of anxiety following CCK administration. In particular, CCK_{B1} receptor activation induces anxiety, while CCK_{B2} agonists increase memory of and/or sustain attention to mild stressors (Dauge & Lena, 1998; Gerhardt et al., 1994 c.f. Harro & Oreland, 1993). The functional impact of the CCK_{B2} receptor was prompted by the observation that BC-264, a selective CCK_B agonist, enhanced memory but did not produce anxiety in a novel environment (Taghzouti et al., 1999) or the elevated plus maze (Ladurelle et al., 1997). In particular, BC-264 improved cue initiated performance among rats in a spontaneous alternation paradigm and a spatial two-trial memory task (Charpentier et al., 1988; Corringer et al., 1992, Ladurelle et al., 1997, Lena et al., 1999). Central hippocampal administration of L-365,260 suppressed novel arm recognition in the spatial two-trial memory task (Sebret et al., 1999). Peripheral administration of BC-264 (3 μg/kg) increased exploration among rats in an open field and such investigatory behaviour was associated with elevated in vivo extracellular concentrations of met-enkephalin, DA, DOPAC and HVA in the rostral nucleus accumbens (Dauge et al., 1999; Ladurelle et al., 1997; Lena et al., 1997). Central administration of naltrindole (0.25 μg /0.5 μl) in the anterior nucleus accumbens suppressed

the increased exploration induced by BC-264 in rats (Dauge et al., 1999). Moreover, central administration of the δ_1 agonist , D-Pen^2, L-Pen^5 (DDLPE) decreased passive avoidance in rats trained to avoid footshock (Ukai et al., 1997). Taken together, these data suggest that activation of accumbal and hippocampal CCK_{B2} receptors promote, while δ_1 receptor activation attenuates saliency of stressor associated cues.

It should be underscored that while functional correlates associated with central activation of a putative CCK_{B2} receptor are evident, (Derrien et al., 1994), anatomical evidence is lacking. However, it appears that mildly aversive stimuli, including exposure to a novel environment or administration of non-anxiogenic doses of CCK, may increase the association of salient cues and environmental context in rats. Such empirical data are consistent with arguments favoring analysis of behavioural sensitization and conditioning following mild stressor presentations, discrete alterations of accumbal and meso-hippocampal DA activity and the induction of learning (Goudie, 1993; Stam et al, 2000; Stewart & Badiani, 1993). Some laboratories have posited that systemic administration of non-anxiogenic doses of CCK peptides facilitates avoidance performance of rats (Hadjiivanova et al., 1994), increases discrimination accuracy (Flood et al., 1995) and accelerates habituation to novelty in open field and hole-board paradigms by increasing attention (Gerhardt et al., 1994). Injection of non-anxiogenic, as defined by performance in the elevated plus maze, doses of CCK-8S or Boc-CCK-4 into the central amygdaloid nucleus enhanced performance of mice trained to avoid footshock (Huston et al., 1998). Systemic administration of the CCK-8S analogue, caerulein or the CCK_B agonist, CCK-8US increased performance of rats trained to avoid footshock. Bilateral 6-OHDA lesions of the central amygdala abolished the facilitatory

effect on performance of CCK-8US and caerulein (Winnicka & Wisniewski, 1999). In a previous investigation, application of β-endorphin into the basolateral amygdala and the ensuing μ receptor activation attenuated CCK enhanced retention of footshock avoidance in rats (Flood et al., 1992). These data suggest that sensitization and conditioning may involve a amygdaloid DA/CCK-opioid colocalization mosaic to encode stressor saliency. Such a proposition would certainly be consistent with an amygdaloid interpretation of emotional memory (Davis et al., 1994; Davis, 1994 and 1998) and negative reinforcement learning (Cahill & McGaugh, 1990). Electrophysiological investigations have revealed that the μ_1 agonist DAGO and the δ_1 agonist DPDPE reduced synaptic transmission in accumbal (Yuan et al., 1992) and hippocampal (Alreja et al., 2000) slices. Data collected in this laboratory revealed that central δ receptor activation following footshock and novelty in CD-1 mice only increased locomotor activity with reexposure to a milder form of the original stressor. The behavioural sequelae of δ receptor activation following stressor imposition were dose and time dependent (Hebb et al., 1997).

In contrast to the memory enhancing properties of non-anxiogenic doses of CCK in anxiety paradigms, CCK-4 and BC-264 inhibit socially reinforced memory (Derrien et al., 1993 and 1994) and olfactory recognition performance (Lemaire et al., 1994). Social interaction and defeat paradigm indices correlate with extracellular met-enkephalin availability in the rostral nucleus accumbens (Bertrand et al., 1997) and μ receptor mRNA density in the VTA (Nikulina et al., 1999), respectively. Indeed, naltrexone increased initial contact latency and decreased active interaction duration among rats in a social interaction paradigm (Zhang et al., 1996). Psychological stressors increase amygdaloid δ receptor binding

(Pohorecky et al., 1999) and μ receptors in the VTA (Nikulina et al., 1999), paralleling anxiety induction in rats and mice (Blanchard et al., 1998; MacNeil et al., 1997; Moynihan et al., 2000). It will be recalled that central administration of enkephalin agonists, prior to or following neurogenic stressors attenuate stressor associated deficits in locomotor activity (Hebb et al., 1997) and brain stimulation (Zacharko et al., 1998) in mice. It is conceivable that BC-264 preserves the memory of a CCK encoded event or a stressor and central μ_1 and δ_1 receptor activation interferes with the encoding of such events. Taken together, alterations in mesolimbic CCK availability may define mild anxiety states. In particular, a μ_1 and δ_1/ CCK_B interface within the VTA, nucleus accumbens and amygdala may diminish anxiety and motivational loss accompanying stressor exposure, permit expression of coping behaviour, detract from the saliency of the stressor and alter long-term responsivity to ensuing stressor encounter.

In summary, the notion that exposure of animals to mild stressors induces behavioural conditioning and sensitization is unequivocal. Indeed, stressful life events and the biochemical concomitants of anxiety alter neuronal activation (i.e., c-fos expression which provides a marker for altered transmitter availability), receptor sensitivity and neuropeptide release which underlie increased behavioural responsivity for protracted intervals. Mild stressors, including novelty and predator odor, promote expression of CCK-8S in the posterior nucleus accumbens and underlie reductions in exploration. At the same time, mild stressors augment CCK-8S in the anterior nucleus accumbens, central and basolateral regions of the amygdala, VTA and mesocortex and presumably provoke motivational alterations consistent with the anxiogenic influence of such manipulations. In particular, increased CCK

availability in the central and basolateral amygdala is ordinarily associated with heightened vigilance and protracted startle. In contrast, increased CCK in the shell of the nucleus accumbens and mesocortex is associated with variations in anxiety and decreased activity in exploration paradigms. In effect, sustained elevation of central CCK has been linked to the protracted impact of stressors on motivation and anxiety in clinical samples.

It should be underscored that enkephalin release within the VTA, in particular, may be a neurochemically relevant cue in the initiation of motivation/reward threshold changes. In human and infrahuman subjects, increased central CCK activity has been associated with anxiety and anhedonia, while increased pro-enkephalin availability is associated with enhanced motivational output. Increased enkephalin availability in humans has been associated with expression of effective coping strategies and mood elevation, while increased CSF CCK has been associated with motivational loss, anticipatory anxiety and panic. In infrahuman subjects, activation of μ_1 and δ_1 receptors in the basolateral amygdala, VTA and mesocortex attenuates the aversiveness of psychogenic stressors. Moreover, CCK_B antagonists and enkephalin agonists have demonstrable antidepressant and anxiolytic properties in infrahuman and human subjects. The temporal expression of enkephalin and CCK in mesolimbic sites associated with specific indices of anxiety may define behaviours associated with mild stressor exposure, including coping, and predict vulnerability to comparable stressor encounter. The propensity of enkephalin to increase mood and affect and detract from the saliency of the stressor experience suggests increased enkephalin availability within the mesencephalon ameliorates protracted CCK-induced motivational alterations assessed in reward paradigms and exploration tasks. In this respect, central

administration of CCK-8S would induce motivational alterations in animals responding for brain stimulation from the VTA which would be ameliorated by μ_1 and δ_1 activation. Taken together, mild stressors evoke gradations of anxiety dependent upon the motivational state of the animal. The behavioural discourse accompanying expression of anxiety will be paralleled by increased expression of cholecystokinin mRNA and decreased enkephalin levels in mesolimbic sites associated with the initiation, maintenance and expression of behavioural change. The temporal expression and duration of such neural changes may reveal specific functionality's of CCK and enkephalin receptor activation to particular symptoms or behavioural subsets associated with clinical pathology.

The series of Experiments described in this dissertation evaluated a) the pattern of the CCK precursor molecule (procholecystokinin) mRNA expression in the medial prefrontal cortex (mPFC), ventral tegmental area (VTA) and the basolateral (BLA) and medial amygdaloid (MEA) nuclei following acute exposure of CD-1 mice to soiled rat shavings in a novel environment b) the propensity of rat odor exposure to induce anxiety in the light-dark box at various intervals following stressor encounter c) acoustic startle among CD-1 mice following acute exposure to rat odor d) the pattern of expression of procholecystokinin mRNA from the mPFC, VTA and the basolateral amygdaloid nuclei following acute exposure of CD-1 mice to fox odor e) the propensity of fox odor exposure to induce defensive behaviours in response to home-cage fox odor presentation as well as anxiety in the light-dark box f) the expression of fos related antigens (FRA) and/or the enkephalin precursor gene (prepro-enkephalin) mRNA from the VTA, mPFC, core and shell of the nucleus accumbens, and basolateral, medial and central amygdaloid nuclei in CD-1 mice following acute exposure

to fox odor and the defensive behavioural manifestations associated with odor presentation in the home-cage and anxiety in the light-dark box g) startle reactivity following acute fox odor presentation h) reward threshold changes from the dorsal aspects of the VTA following acute exposure to fox odor h) the immediate and protracted effects of an acute administration of CCK-8S or Boc CCK-4 on conditioned fear in CD-1 mice following footshock in a fear potentiated startle paradigm i) the immediate and long-term behavioral effects of central administration of the anxiogenic substance, CCK-8S on threshold determinations from sub-regions of the VTA and locomotor and rearing in CD-1 mice and finally j) the propensity of the μ/δ enkephalin agonist DALA to ameliorate behavioral deficits in CD-1 mice to an acute 50 ng CCK-8S dose as well as to a challenge dose (i.e., 5 ng) of CCK (i.e., behavioural sensitization) administered 18 days following initial CCK administration.

EXPERIMENTS 1 AND 2

Uncontrollable stressors have been associated with the provocation and exacerbation of psychological disturbance, including anxiety (see Zacharko et al., 1995). In particular, various laboratories have employed psychogenic stressors (e.g., predator exposure, and predator associated cues) to elicit behavioural disturbances and induce neurochemical perturbation. For example, visual, olfactory, auditory and tactile features of feline encounter enhance acoustic startle, avoidance, ultrasonic vocalization, freezing and defensive threat attack behaviours in rats in comparison to animals which were only exposed to the predator odor (Adamec et al., 1999; Blanchard et al., 1998; Dielenberg et al., 1999). Such behavioural profiles are also evident among mice exposed to a rat (Blanchard et al., 1998) and the

flight/avoidance behaviour of mice and rats to such predatory encounter has been offered as an animal model of panic disorder (Griebel et al., 1996).

Some investigators have partitioned the anxiogenic features associated with predator exposure. For example, a 5 minute exposure of independent groups of rats to the odor of a cat decreased social interaction duration as well as open arm occupancy and open arm entries in the elevated plus maze. Behavioural evidence for augmented anxiety in the social interaction and elevated plus maze paradigms was conspicuous 35-60 minutes following cat odor exposure but not 24 hours following initial odor encounter (Zangrossi & File, 1992). In contrast, acute 5 minute exposure of rats to a cat increased acoustic startle immediately and anxiety in the elevated plus maze 30-60 minutes following exposure and such behavioural alterations persisted 7 and 21 days post-stressor encounter, respectively (Adamec et al., 1997; Adamec et al., 1998; Adamec & Shallow, 1993). In an ensuing paradigm, anxiety among rats in the elevated plus maze 7 days following cat exposure was antagonized by prophylactic and attenuated by therapeutic administration of the CCK_B receptor antagonist, PD 135,158, 30 minutes prior or 30 minutes following predator exposure (Adamec et al., 1997). In a similar vein, a 15 minute exposure of rats to cat scent increased CCK-4 concentrations in the mesocortex, nucleus accumbens and central amygdaloid nucleus immediately following predator odor. Such neurochemical variations were associated with enhanced instances of freezing relative to animals which were exposed to a neutral scent (Pavlasevic et al., 1993). Anxiogenic responses of rats to predator cues do not appear to habituate (Zangrossi & File, 1992), generalize to neutral odors (e.g., clean shavings) (Zangrossi & File, 1994), promote behavioural sensitization to d-amphetamine challenge (Williams & Barber, 1990) and are attenuated by benzodiazepine administration (Barros et

al., 2000; McGregor & Dielenberg, 1999). In addition to exposure of rats to the scent of a cat, defensive and anxiety behaviours of mice to the scent of rat, red fox, stoat and owl have also been investigated (De Catanzaro, 1988; Griebel et al., 1995; Hendrie & Dourish, 1993; Hendrie et al., 1996; Hotsenpiller & Williams, 1997; Kavaliers et al., 1997; Kavaliers & Choleris, 1997). Such diverse predator stressors may provide distinct cues (e.g., owl vocalization cf. fox odor) which may define prey-predator relationships and impact on anxiety gradients.

It will be recalled that merely exposing rats to predator odor enhances anxiety in the elevated plus maze and social interaction paradigms for relatively abbreviated intervals (Zangrossi & File, 1992) and increases mesolimbic CCK-4 concentrations immediately following odor encounter (Pavlasevic et al., 1993). Consistent with such a demonstration, Experiment 1 evaluated behaviour among CD-1 in the light-dark task and the pattern of mesolimbic CCK mRNA in the basolateral and medial amygdaloid nucleus as well as the mesocortex and the VTA following acute natural predator odor exposure (e.g., rat). The light dark task, rather than the elevated plus maze, was employed owing to the reliability, validity and simplicity of the light dark box paradigm (Chaouloff et al., 1997; Costall et al., 1989). In addition, simple manipulation of arena intensity (e.g., 60 cf. 40 watt) augments or diminishes basal anxiety levels (Costall et al., 1989). Indeed, such manipulations have been employed in this laboratory to assess anxiogenic behaviour of CD-1 mice exposed to footshock, restraint and central CCK-8S administration (MacNeil et al., 1997). Experiment 2 assessed acoustic startle among CD-1 mice following rat odor exposure. Acoustic startle was included as an anxiogenic measure responsive to repeated testing (Gewirtz et al., 1998). In contrast, repeated exposure of animals to the light-dark box or the elevated plus maze preclude

behavioural evaluation and anxiolytic benzodiazepine influence (File, 1993; File & Zangrossi, 1993; File et al., 1998). Nevertheless, is conceivable that CCK availability prompted by specific or repeated stressor encounter enhances stressor saliency. In effect, it is suspected that such anxiogenic experience modifies behaviours ordinarily associated with anxiety (i.e., light-dark paradigm) and presumably fear (i.e., startle paradigm) prompted by increased expression of CCK in distinct areas of the mesolimbic system.

MATERIALS AND METHODS

Subjects

One hundred and thirty, naive, male, CD-1 mice (Charles River, St Constant, Quebec) were group housed (5 weeks of age) with ad libitum access to food and water, and maintained on a 12 hour light-dark schedule. Mice were approximately six months old at time of testing. Two to three weeks prior to behavioural testing, mice were individually housed and handled daily. Handling consisted of picking up animals gently by the tail and allowing free exploration of a gloved hand for 2 minutes. All behavioural testing was conducted diurnally in laboratory quarters removed from the animal housing area. The Carleton University Animal Care Committee approved the Experiments described in this dissertation and the protocols were in accordance with the guidelines detailed by the Canada Council on Animal Care.

Odor

Exposure of mice to the odor of a predator was accomplished by placing a mouse in a rectangular Plexiglas cage (25 x 45 x 20 cm) lined with soiled rat bedding (predator odor, PO). An identical cage lined with clean wood shavings served as the non-predatory control (control odor; CO). All animals were returned to the home cage following odor exposure. A third

group of handled home cage mice (home cage odor, HC), which were neither exposed to the predator odor nor the clean wood shavings served as the no odor control treatment condition.

Light-Dark Paradigm

The light-dark apparatus consisted of a rectangular Plexiglas box (20 x 47 x 20 cm) with the black section comprising one-third and the white section comprising the remaining two-thirds of the chamber. The two sections of the apparatus were separated by a Plexiglas partition with an opening (12.5 x 5 cm) allowing passage from the open "white" section to the enclosed "black" portion of the chamber. A 40-watt light bulb, positioned 10 cm above the center of the white compartment, provided illumination, while red, translucent Plexiglas served as a roof of the black chamber. Mice were habituated to the rectangular Plexiglas cage (25 x 45 x 20 cm) lined with clean bedding for 10 minutes on three consecutive days. On each trial mice were transported to the test site which housed the light-dark box apparatus. Mice were not exposed to the light-dark box during the habituation procedure. The test day experimental protocol was simulated on each of the 3 habituation days by gently removing each mouse from the home cage and handling subjects briefly (2 minutes) before returning each animal to the home cage and transporting mice back to the main animal housing unit.

Following termination of the habituation protocol, mice were randomly assigned to either the CO or PO treatment conditions (n=25/group) and behaviour was evaluated in the light-dark task immediately (CO n=5, PO n=5), 30 minutes (CO n=5, PO n=5), 1 hour (CO n=5, PO n=5), 2 hours (CO n=5, PO n=5) or 4 hours (CO n=5, PO n=5) following odor exposure. Animals in the HC (n=5) condition were tested immediately in the light-dark box following home cage removal . Each subject was placed in the center of the light compartment facing the dark chamber entry. The latency for the mouse to enter the dark chamber, transition

frequency between the white and black compartments as well as cumulative light and dark compartment occupancy were recorded over two consecutive 5 minute sessions. A subject was considered to have entered a compartment when all four paws were positioned within that compartment. A Samsung SCX954 video camera positioned above the light-dark box recorded behaviour of mice. Three animals from each treatment condition were perfused intracardially with 4% paraformaldehyde (3.8% Sodium Borate) and brains removed for in situ hybridization of preproCCK mRNA immediately following light-dark box exposure.

In Situ Hybridization

A specific cDNA probe was obtained by DNA amplification starting from rat cerebral cortex RNA. Total RNA was isolated from rat brain using the guanidine isothyocianate method (Chomezinski & Sacchi, 1987). Briefly, brain tissue was homogenized at 4°C in a buffer containing 50 mM Tris-HCI (pH 7.5), 4 M guanidine isothiocyanate, 10mM EDTA, N-laurylsarcosine (1% wt/vol), and 2-mercaptoethanol (1% vol/vol). The samples were extracted twice with phenol/chloroform (1:1 vol/vol) and the nucleic acids were recovered by precipitation with ethanol. DNAse treatment of the nucleic acids was followed by a further extraction with phenol/chloroform (1:1 vol/vol) and RNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nanometers. For direct DNA amplification, 1 µg of cortex RNA was used. Reaction was performed using Titan One tube RT-PCR system (Roche) and primers at 400 µm each in 50 µl reaction tube. The parameters for DNA amplification were 30 minutes at 42°C for reverse transcriptase, 94°C for 2 minutes, annealing temperatures for 30 seconds and 68°C for 7 minutes, where the annealing temperature was optimized for each pair of primers. The sense primer 1 was 5'CCG-CCT-

GCC-CTC-AAC-TTA 3' position 40-58 a/s and primer 2 was 5'AAA-CAT-TGC-CTT-CCC-ACC 3' position 644-626. DNA amplification products were analyzed by gel electrophoresis on an agarose gel (0.8% wt/v), which was stained with ethidium bromide. To subclone amplified DNA, the polymerase chain reaction (PCR) product was purified, digested with appropriate restriction enzymes and ligated to a similarly digested sample of p Blue script SK (+/-) (Promega). To produce the anti-sense probe, DNA was digested with Ecor1 and T3 polymerase. To produce the sense probe, DNA was digested with Sac1 and T7 polymerase. Hybridization with the sense probe did not reveal any positive signal in mouse brain.

Light-Dark Box Data Analyses

Behavioural scores describing dark compartment latency, time in light during the initial and subsequent 5 minute trial block as well as cumulative time in light and transitions between the light and dark compartments were analyzed by one-way analyses of variance for independent groups following stressor encounter. Fisher's Least Significant Difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

Quantitative Analysis of CCK mRNA

Nuclei were identified using the mouse brain atlas of Paxinos and Franklin (1997). For each mouse, amygdaloid sections corresponding to level 43-44, the medial prefrontal cortex corresponding to levels 14-19 and the VTA corresponding to levels 58-62 were examined. Blind quantitative analysis of hybridization signal for CCK mRNA was performed on Kodak X-ray film. The signal was analyzed with NIH image software. Densitometric analyses, yielding measures of optical density, area of signal and integrated optical (i.e., area of the

signal x average optical density) were performed according to a standard scale established using ^{14}C standard slides. The density of the respectively identified cell types (i.e., positively stained cells) evaluated in the basolateral amygdaloid nucleus (BLA), medial amygdaloid nucleus (MEA), ventral tegmental area (VTA) and mesocortex (mPFC) were subjected independently to a one-way ANOVA for independent groups following odor encounter to facilitate identification of relevant stressor intervals on CCK induced changes in mesolimbic sites.

RESULTS

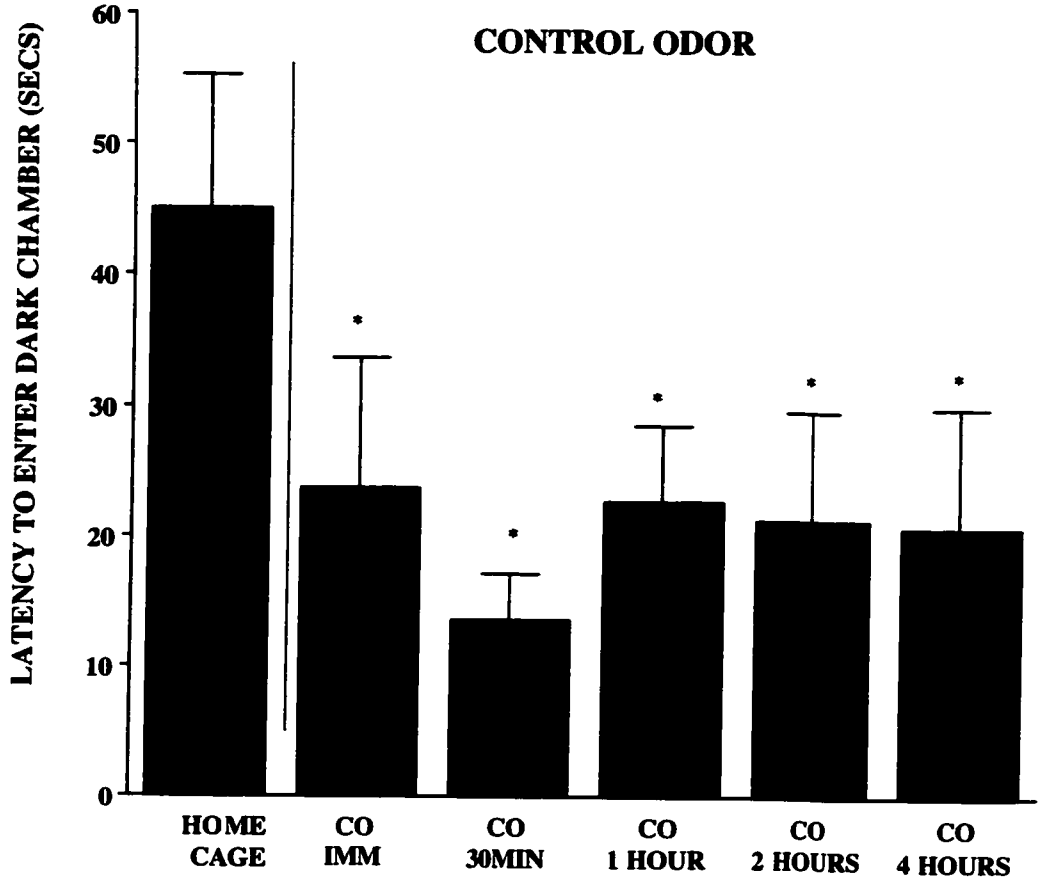
Light-Dark Box Behavioural Measures

Analysis of variance of the latency to enter the dark compartment of the light-dark box among mice following odor exposure revealed a significant main effect of Treatment Condition, $F(10,43)=2.12$, $p<.05$. Fisher's LSD revealed mice exposed to either clean shavings or rat bedding displayed a decreased latency to enter the dark compartment of the light-dark box relative to home cage housed mice immediately, 30 minutes, 1 hour, 2 hours and 4 hours following odor presentation (see Figure 1.1).

Insert Figure 1.1 about here

Analysis of variance of time spent in the light in the light-dark paradigm during the first and subsequent 5 minute trial blocks among mice following odor exposure revealed a significant main effect of Treatment Condition, $F's\ (10,43)=2.15$ and 2.25, $p<.05$, respectively. During the initial 5 minute trial block, Fisher's LSD multiple comparisons revealed that mice exposed to clean shavings spent significantly less time in the light

Figure 1.1: Mean (± S.E.M.) latency to enter the dark compartment among mice following exposure to a novel environment (CO) (top panel) or rat odor (PO) (Bottom panel) immediately, 30 minutes, 1 hour, 2 hours or 4 hours following odor presentation relative to home-caged (HC) mice. Note: * depicts a statistically significant difference ($p < .05$) of CO or PO odor treatment relative to HC mice.


CONTROL ODOR
LATENCY TO ENTER DARK CHAMBER (SECS)
60
50
40
30
20
10
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HOME CAGE
CO IMM
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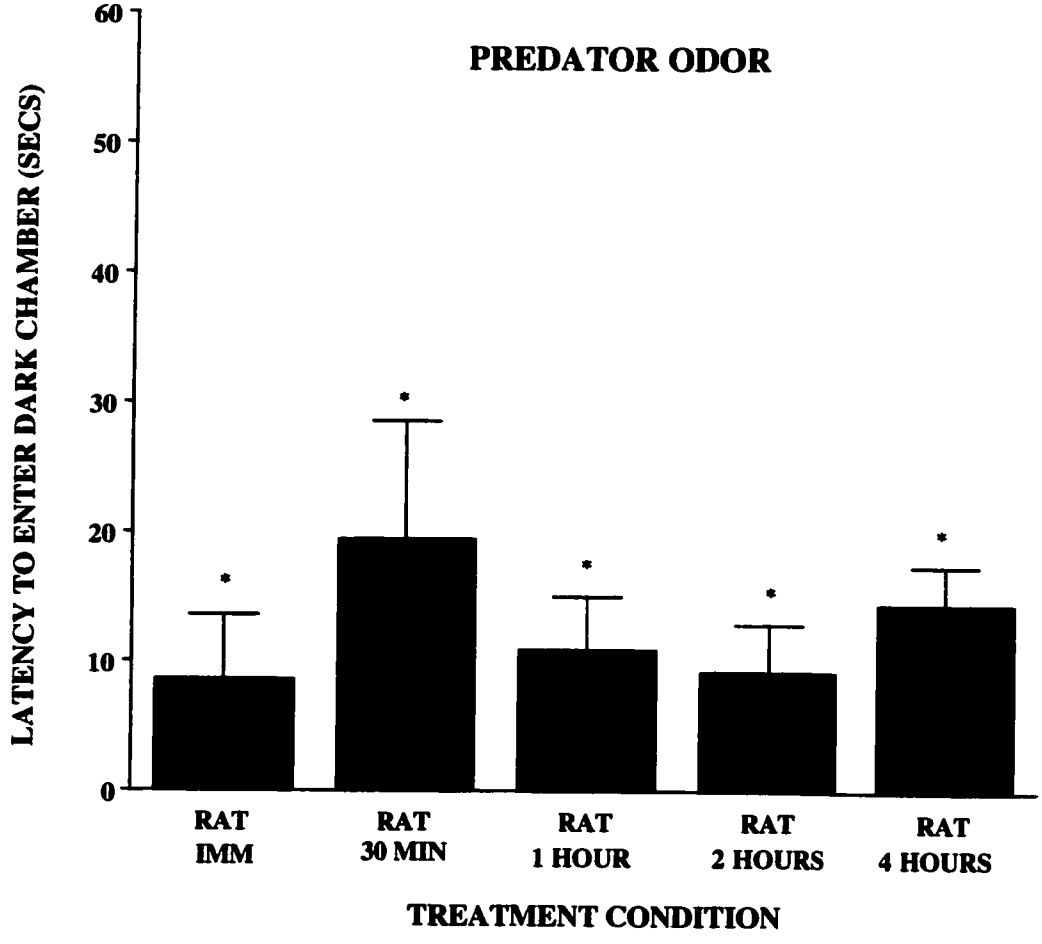
CO 1 HOUR
CO 2 HOURS
CO 4 HOURS


PREDATOR ODOR
LATENCY TO ENTER DARK CHAMBER (SECS)
60
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RAT IMM
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RAT 1 HOUR
RAT 2 HOURS
RAT 4 HOURS
TREATMENT CONDITION

compartment of the light-dark box relative to home cage housed mice, 30 minutes, 2 hours and 4 hours following odor encounter. Mice exposed to rat bedding spent significantly reduced time in the light compared to home cage odor mice immediately, 2 hours and 4 hours following odor presentation. Fisher's LSD multiple comparisons also revealed that CO mice spent significantly more time in the light 1 hour following odor exposure relative to CO mice evaluated 2 or 4 hours following odor presentation. Mice exposed to the predator odor spent significantly less time in the light in the immediate post-stressor interval relative to predator odor exposed mice evaluated in the paradigm 30 minutes or 1 hour following odor presentation. Control mice spent significantly more time in the light relative to predator odor exposed mice immediately following odor presentation.

During the subsequent 5 minute trial block, Fisher's LSD revealed that mice exposed to clean bedding spent significantly less time in light relative to home caged housed animals 30 minutes, 2 hours as well as 4 hours following bedding exposure. Mice exposed to rat odor bedding spent significantly less time in the light compared to home caged mice immediately, 30 minutes and 4 hours following odor presentation. Fisher's LSD multiple comparisons also revealed that mice exposed to clean shavings spent significantly more time in the light 1 hour post-odor exposure relative to clean shaving exposed mice 30 minutes, 2 hours or 4 hours following odor encounter. Mice in the predator odor treatment group evaluated immediately following rat bedding exposure spent significantly less time in the illuminated arena relative to mice exposed to predator odor 1 hour following stressor presentation. Mice housed in home cages spent significantly more time in the light compared to predator odor treated mice 1 hour following stressor presentation (see Figure 1.2).

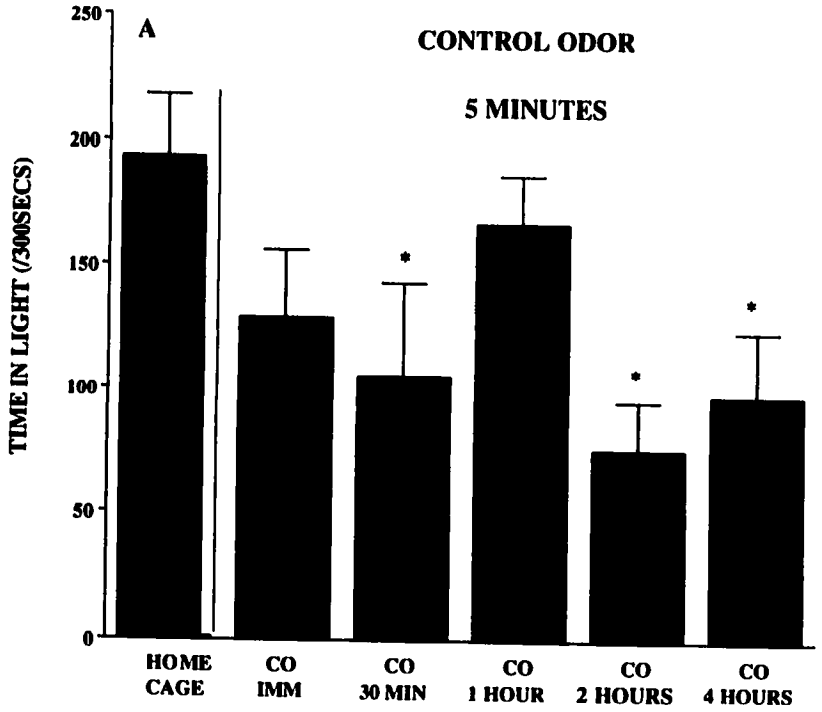
Insert Figure 1.2 about here

Analysis of variance of cumulative time in the light chamber of the light dark box among mice following odor exposure revealed a significant effect of Treatment, $F(10,43) = 2.48$, $p < .05$. Fisher's LSD revealed that mice exposed to clean shavings spent significantly less time in the light compared to home cage housed mice immediately, 30 minutes, 2 hours and 4 hours following odor presentation. Fisher's LSD also revealed that mice exposed to the rat odor spent significantly less time in the illuminated arena compared to home cage housed mice immediately, 30 minutes, 2 hours and 4 hours following odor presentation. Fisher's LSD multiple comparisons revealed that mice exposed to clean shavings spent significantly more time in the light 1 hour post-stressor encounter relative to home caged assigned mice immediately, 30 minutes, 2 hours or 4 hours following odor presentation. The cumulative time spent in the light among mice exposed to rat bedding did not vary significantly with the post-odor sessions examined (see Figure 1.3).

Insert Figure 1.3 about here

Analysis of variance of transition frequency between the light and dark chambers of the light-dark box during the initial and subsequent 5 minute trial blocks intervals as well as the cumulative transitions among mice following odor exposure failed to reveal significant differences among treatment conditions, F's $(10,43) = 1.04$, 1.69, and 1.13, $p > .05$, respectively (data not shown).

Figure 1.2: Mean (± S.E.M.) time in light during the first (right) and second (left) consecutive 5 minute intervals among mice following exposure to a novel environment (CO) (Figures 1.2 A and 1.2 B) or rat odor (PO) (Figures 1.2 C and 1.2 D) immediately, 30 minutes, 1 hour, 2 hours or 4 hours following odor presentation relative to home-caged (HC) mice. Note: * depicts a statistically significant difference ($p < .05$) of CO or PO odor treatment relative to HC mice while ** depicts a statistically significant difference ($p < .05$) of CO and PO odor treatments at the identical post-stressor interval.


A
CONTROL ODOR
5 MINUTES
TIME IN LIGHT (/300SECS)
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200
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50
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HOME CAGE
CO IMM
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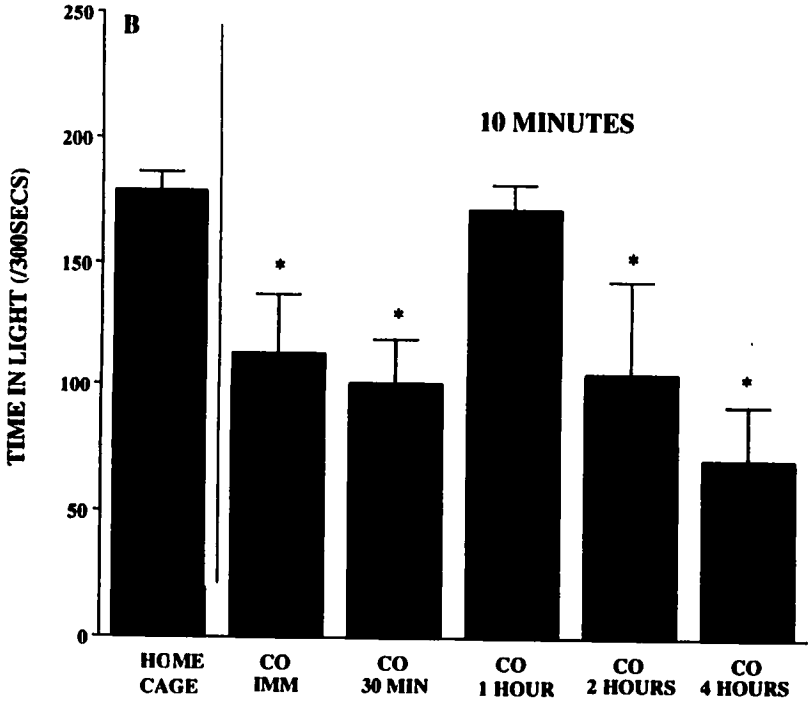
CO 2 HOURS
CO 4 HOURS


B
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TIME IN LIGHT (/300SECS)
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CO 1 HOUR
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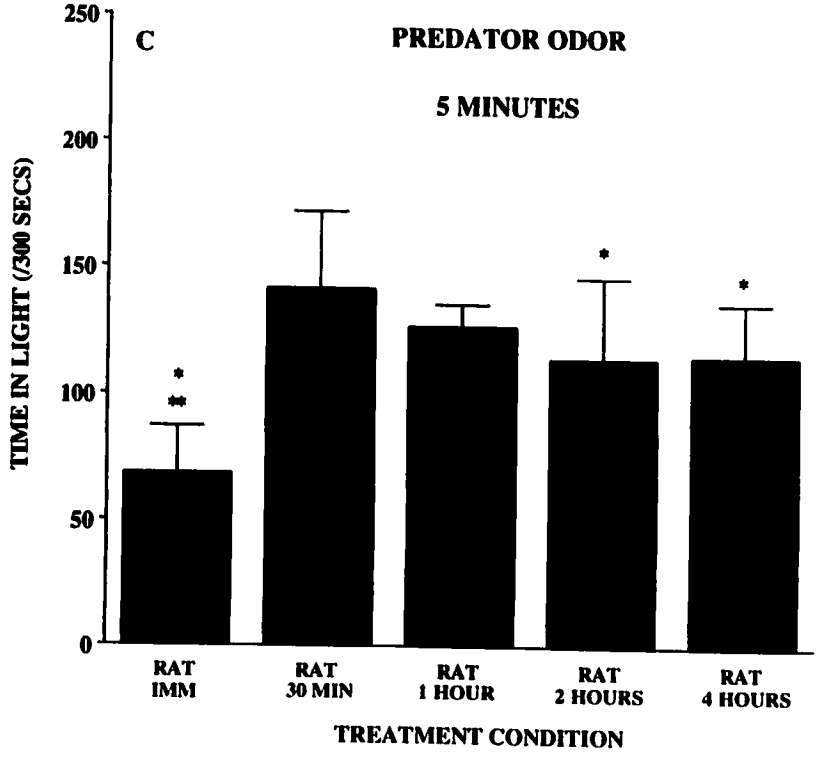
CO 4 HOURS


C
PREDATOR ODOR
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TIME IN LIGHT (/300 SECS)
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RAT IMM
RAT 30 MIN
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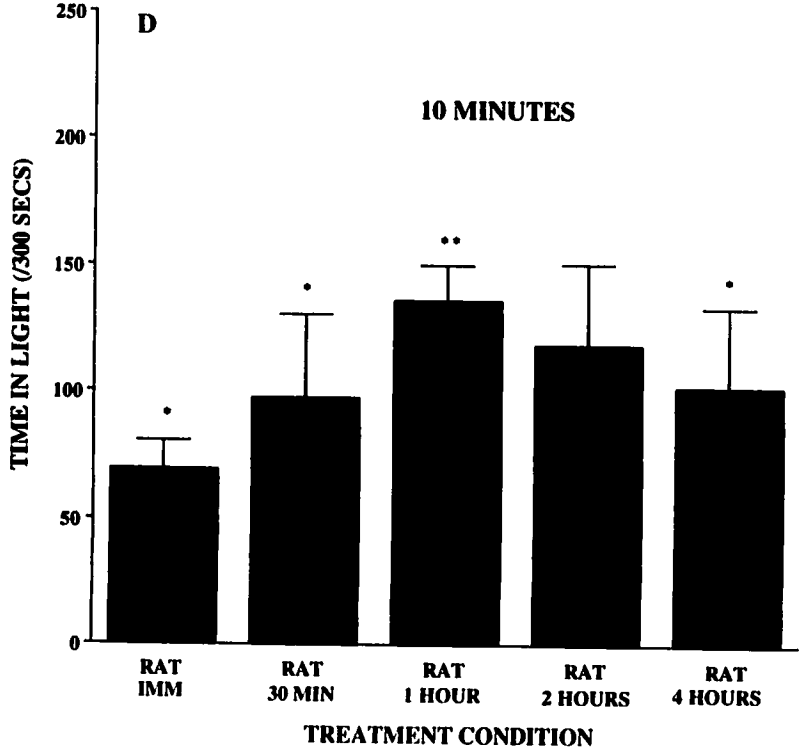
RAT 2 HOURS
RAT 4 HOURS
TREATMENT CONDITION


D
10 MINUTES
TIME IN LIGHT (/300 SECS)
250
200
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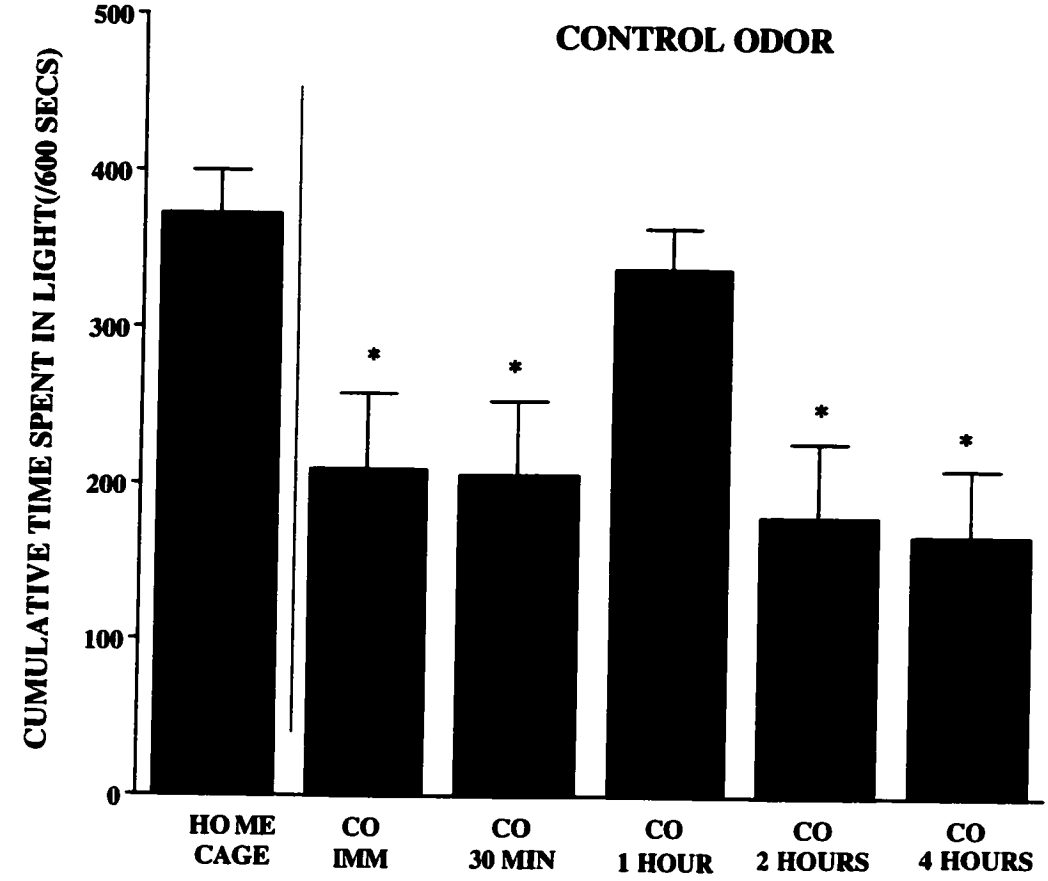
100
50
0
RAT IMM
RAT 30 MIN
RAT 1 HOUR
RAT 2 HOURS
RAT 4 HOURS
TREATMENT CONDITION

Figure 1.3: Mean (± S.E.M.) cumulative time in light among mice following exposure to a novel environment (CO) (top panel) or rat odor (PO) (bottom panel) immediately, 30 minutes, 1 hour, 2 hours or 4 hours following odor presentation relative to home-caged (HC) mice. Note: * depicts a statistically significant difference ($p < .05$) of CO or PO odor treatment relative to HC mice.


CONTROL ODOR
CUMULATIVE TIME SPENT IN LIGHT(/600 SECS)
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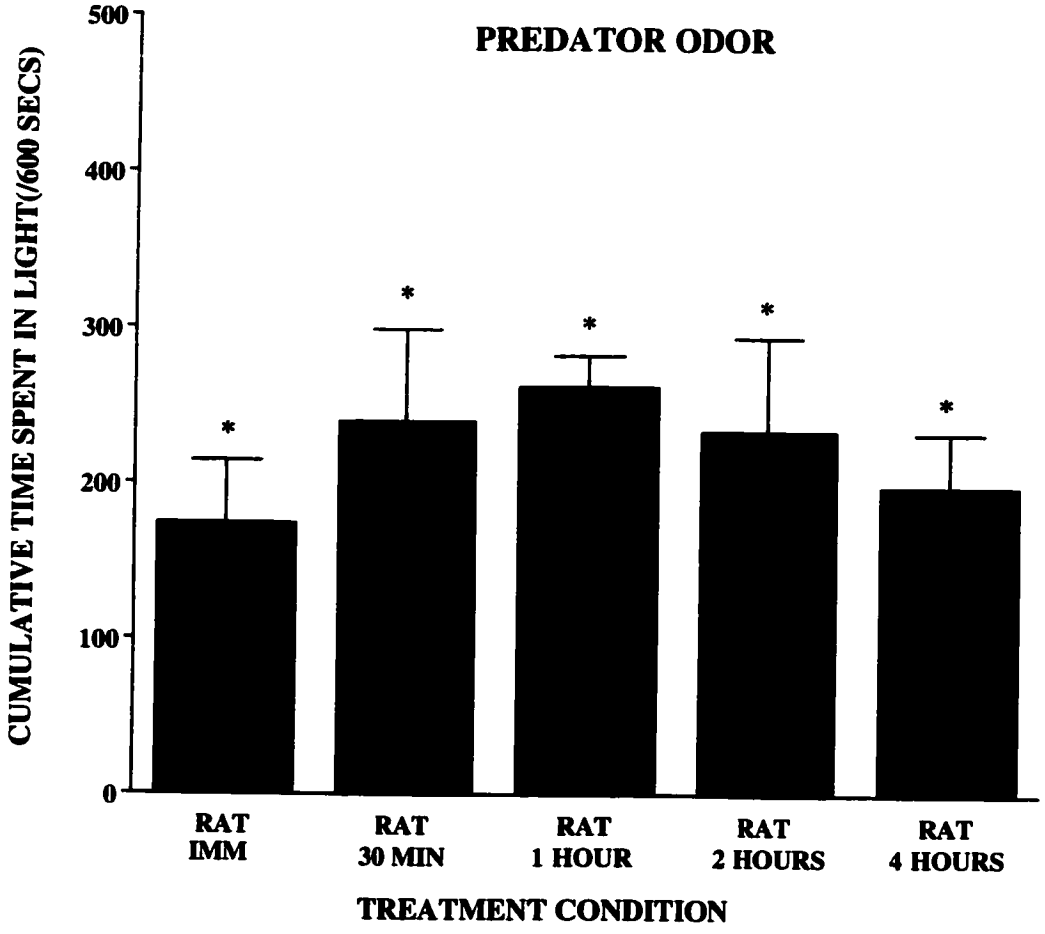
300
200
100
0
*
HO ME CAGE
CO IMM
CO 30 MIN
CO 1 HOUR
CO 2 HOURS
CO 4 HOURS


PREDATOR ODOR
CUMULATIVE TIME SPENT IN LIGHT(/600 SECS)
500
400
300
200
100
0
*
RAT IMM
RAT 30 MIN
RAT 1 HOUR
RAT 2 HOURS
RAT 4 HOURS
TREATMENT CONDITION

Densitometric Analyses of CCK mRNA

Medial Prefrontal Cortex (mPFC)

Analysis of variance of CCK mRNA optical density in the mPFC among mice following odor exposure and light dark box activity revealed a significant main effect of Treatment Condition, $F(10,22) = 2.36$, $p<.05$. Mice exposed to clean shavings (CO) displayed significantly elevated mPFC CCK mRNA relative to home caged housed mice (HC) immediately (0.5 hours) following stressor encounter. Mice demonstrated basal mPFC CCK mRNA levels 1.0 hour post-stressor but significantly enhanced CCK mRNA was again evident 1.5 hours, 2.5 hours and 4.5 hours following stressor imposition relative to HC mice. CCK mRNA was elevated among mice exposed to rat bedding (PO) 1 hour post-stressor relative to either the home odor (HC) caged mice or CO mice and this effect was evident 4.5 hours following stressor termination relative to HC mice (see Figure 1.4).

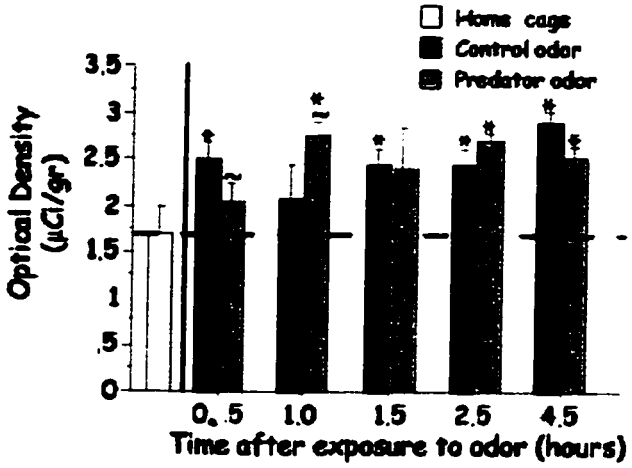
Insert Figure 1.4 about here

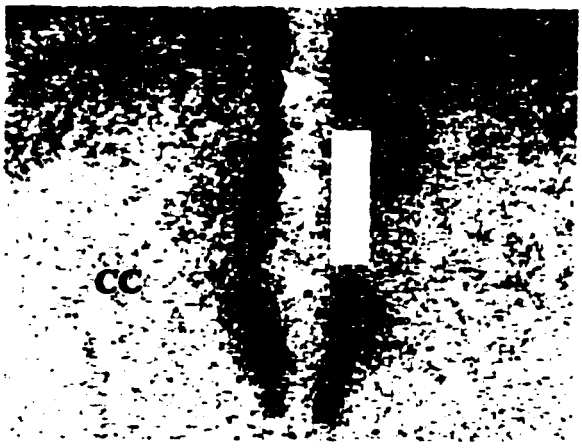
Ventral Tegmental Area (VTA)

Analysis of variance of CCK mRNA integrated density among mice following odor exposure and light dark box activity revealed a significant main effect of Treatment, $F(10,22) = 2.83$, $p< .05$. Mice exposed to clean shavings (CO) displayed significantly elevated CCK mRNA relative to home odor caged mice (HC) and mice exposed to rat bedding (PO) immediately (0.5 hours) following stressor presentation. Such elevated VTA CCK mRNA expression among CO mice returned to basal values 1 hour post-stressor but a significant increase in VTA CCK mRNA expression was again detected 1.5 hours, 2.5 hours and 4.5

Figure 1.4: Cell mean (± S.E.M.) and representative photomicrographs for optical density (μCi/gr) of mPFC CCK mRNA among mice following exposure to a novel environment (CO) or rat odor (PO) relative to home-caged (HC) mice. Note: * depicts a statistically significant difference ($p < .05$) of CO or PO odor treatment relative to HC mice while ~ depicts a statistically significant difference ($p < .05$) of CO and PO odor treatments at the identical post-stressor interval. The time after exposure to odor refers to the time the animal was perfused following behavioral testing in the light-dark box immediately (0.5 hours), 30 minutes (1.0 hour), 1 hour (1.5 hours), 2 hours (2.5 hours) or 4 hours (4.5 hours) following a 10 minute odor presentation. Note:CC= corpus callosum, CG=cingulate cortex, PL=prelimbic cortex, IL=infralimbic cortex, DP=dorsal peduncular area, white rectangle=representative sample area.

Prefrontal Cortex
CCK mRNA





hours following stressor application relative to home cage housed mice (HC). Among mice exposed to rat bedding (PO), significant elevation of CCK mRNA was detected 1 hour post-odor. The augmented VTA CCK mRNA among the predator odor exposed mice returned to basal values 1.5 hours following stressor encounter but CCK mRNA expression was again augmented significantly 2.5 hours and 4.5 hours following stressor application relative to HC mice. The increase in CCK mRNA was most pronounced among PO mice 1 hour following odor presentation relative to CO, HC or PO mice (see Figure 1.5).

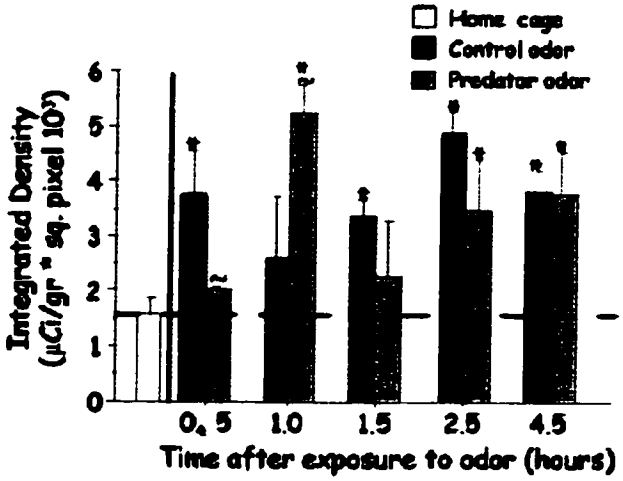
Insert Figure 1.5 about here

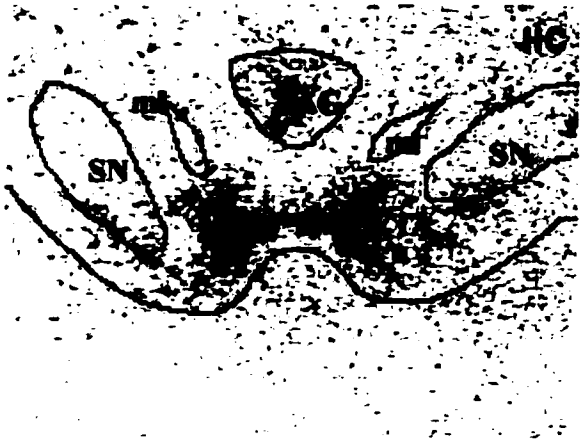
Basolateral Amygdala (BLA)

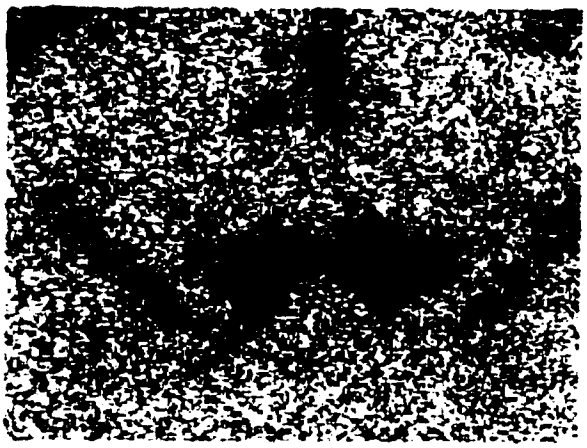
Analysis of variance of CCK mRNA integrated density among mice following odor exposure and light dark box activity revealed a main effect of Treatment, $F(10, 20) = 4.25$, $p<.05$. Mice exposed to clean shavings displayed elevated CCK mRNA levels relative to home cage housed mice, immediately (0.5 hours), 1 hour and 4.5 hours following odor exposure. Mice exposed to the clean shavings in a novel environment displayed increased BLA CCK mRNA values relative to predator odor exposed mice, 1.5 hours and 4.5 hours following odor presentation. There were no significant differences in BLA CCK mRNA among CO mice at any time following odor exposure. Mice exposed to the predator odor shavings displayed significantly elevated BLA CCK mRNA levels immediately (0.5 hours) post-stressor but this effect was no longer evident when assessed 1 hour post-odor presentation relative to HC mice (see Figure 1.6).

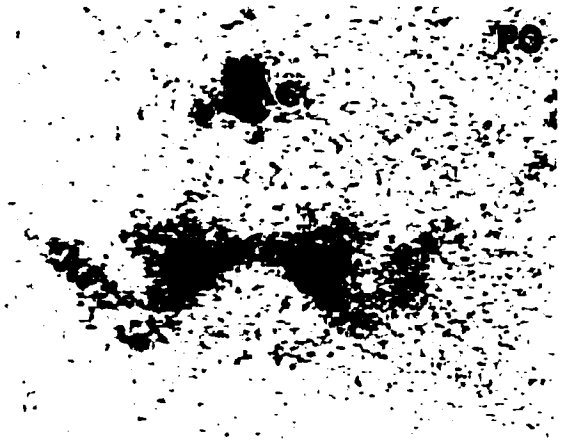
Figure 1.5: Cell mean (± S.E.M.) and representative photomicrographs for integrated density (μCi/gr * sq. pixel 10^3) of VTA CCK mRNA among mice following exposure to a novel environment (CO) or rat odor (PO) relative to home-caged (HC) mice. Note: * depicts a statistically significant difference ($p < .05$) of CO or PO odor treatment relative to HC mice while ~ depicts a statistically significant difference ($p < .05$) of CO and PO odor treatments at the identical post-stressor interval. The time after exposure to odor refers to the time the animal was perfused following behavioral testing in the light-dark box immediately (0.5 hours), 30 minutes (1.0 hour), 1 hour (1.5 hours), 2 hours (2.5 hours) or 4 hours (4.5 hours) following a 10 minute odor presentation. Note: VTA=ventral tegmental area, SN= substantia nigra, PAG= periaqueductal gray area, IF= interfascicular nucleus, ml= medial lemniscus.

Ventral Tegemental Area
CCK mRNA









Insert Figure 1.6 about here

Medial Amygdaloid Nucleus (MEA)

Analysis of variance of CCK mRNA optical density among mice following odor exposure and light dark box activity revealed a main effect of Treatment $F(10, 21) = 4.05$, $p<.01$. Both CO and PO treated mice displayed elevated MEA CCK mRNA relative to HC treated mice at each of the post-stressor intervals examined. There were no significant differences detected in MEA CCK mRNA among CO or PO treated animals at any of the intervals evaluated following odor presentation (see Figure 1.7).

Insert Figure 1.7 about here

Experiment 2

Startle reflex was measured in a ventilated, sound attenuated acoustic chamber (51 x 55 x 31 cm) (Med Associates). Each mouse was placed in an acrylic animal holder (3 x 9 cm) mounted on a response platform. Movement of the mouse within the holder displaced the response platform and generated a digitized analog signal interfaced to an Pentium II computer. Background noise (50 dB) and the acoustic startle stimulus were delivered by a speaker mounted 10 cm above the animal. The startle stimulus was a white noise burst of 110

Figure 1.6: Cell mean (± S.E.M.) and representative photomicrographs for integrated density (μCi/gr * sq. pixel 10^3) of BLA CCK mRNA among mice following exposure to a novel environment (CO) or rat odor (PO) relative to home-caged (HC) mice. Note: * depicts a statistically significant difference ($p < .05$) of CO or PO odor treatment relative to HC mice while ~ depicts a statistically significant difference ($p < .05$) of CO and PO odor treatments at the identical post-stressor interval. The time after exposure to odor refers to the time the animal was perfused following behavioral testing in the light-dark box immediately (0.5 hours), 30 minutes (1.0 hour), 1 hour (1.5 hours), 2 hours (2.5 hours) or 4 hours (4.5 hours) following a 10 minute odor presentation.

Basolateral Amygdala
CCK mRNA

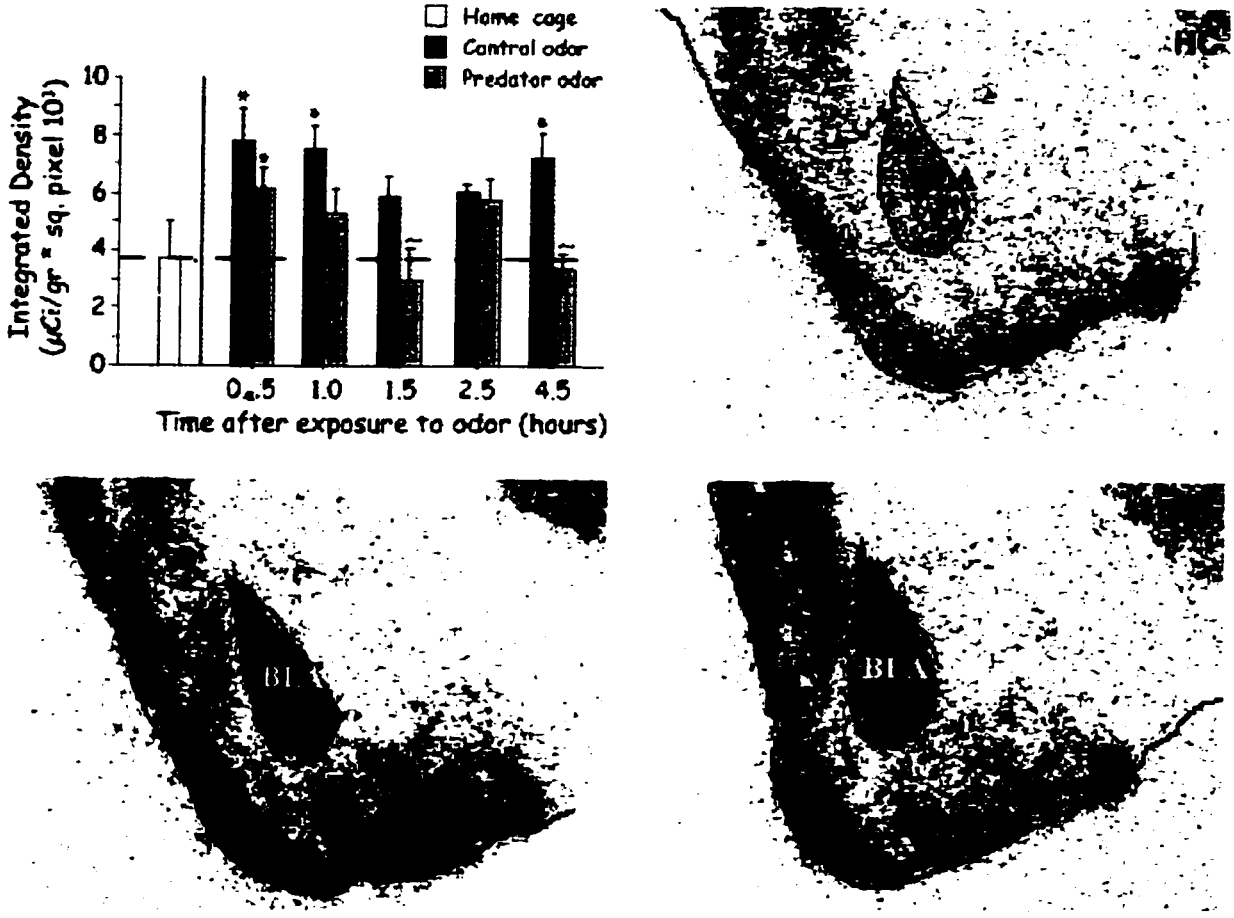
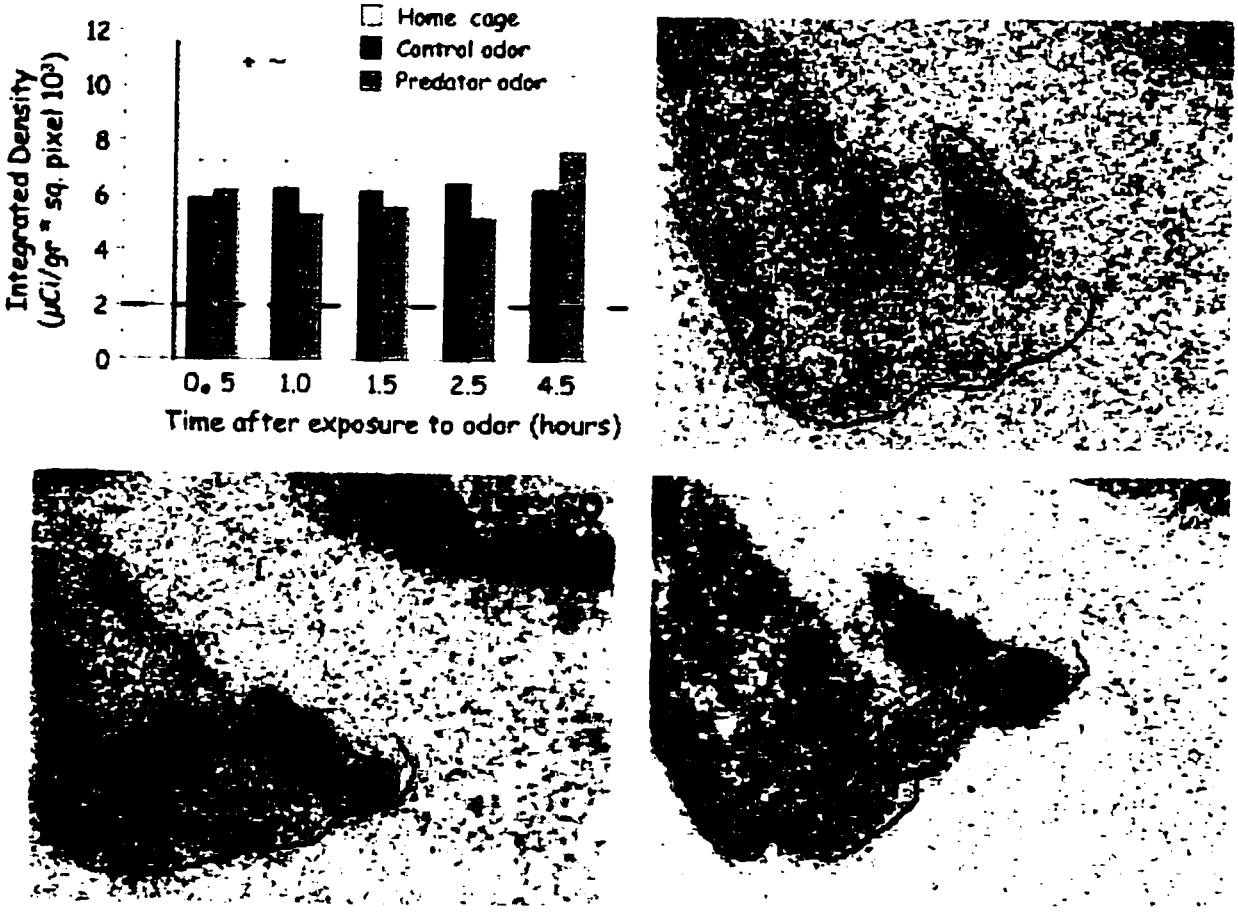


Figure 1.7: Cell mean (± S.E.M.) and representative photomicrographs for integrated density of MEA CCK mRNA among mice following exposure to a novel environment (CO) or rat odor (PO) relative to home-caged (HC) mice. Note: * depicts a statistically significant difference ($p < .05$) of CO or PO odor treatment relative to HC mice. The time after exposure to odor refers to the time the animal was perfused following behavioral testing in the light-dark box immediately (0.5 hours), 30 minutes (1.0 hour), 1 hour (1.5 hours), 2 hours (2.5 hours) or 4 hours (4.5 hours) following a 10 minute odor presentation.

Medial Amygdala
CCK mRNA



dB of 50 ms duration in the absence of a rise-decay period. The startle amplitude was defined as the peak voltage within 150 msec following startle stimulus onset.

All mice were habituated to a rectangular Plexiglas cage (25 cm x 45 cm x 20 cm) lined with clean bedding for 10 minutes on three consecutive days. On each habituation trial mice were transported to a test area where startle responses were measured. Mice were placed in the startle apparatus and following a 5 minute acclimatization period baseline startle was assessed over 10 trials with a variable inter-trial interval set at 25-30 seconds. Following habituation, mice were randomly assigned in Experiment 2A to 10 minutes of CO or PO (n=20/group). Startle was assessed immediately (CO n=4, PO n=4), 30 minutes (CO n=4, PO n=4), 1 hour (CO n=4, PO n=4), 2 hours (CO n=4, PO n=4), or 4 hours (CO n=4, PO n=4) following odor encounter. Identification of protracted alterations in startle reactivity among CD-1 mice was accomplished by assessing startle 24, 48 and 168 hours following odor presentation. A second group of mice (N=35) (Experiment 2B) were assigned to either a CO or PO group and exposed to a 2 minute, 5 minute or 10 minute odor session. Startle reactivity was assessed immediately, 24, 48 and 168 hours following odor presentation. Mice assigned to the home cage odor condition (n=5) served as control subjects. All animals were sacrificed using CO_2.

Behavioural Analysis of Acoustic Startle

Experiment 2A

Startle scores were converted to a percentage of Baseline and subjected to ANOVA assessing Treatment Condition (PO and CO) with repeated measures over days. Fisher's Least Significant Difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

Experiment 2B

Startle scores were converted to a percentage of Baseline and subjected to analysis of variance (ANOVA) as a Treatment Condition (PO, CO or HC) by Time (2 minutes, 5 minutes or 10 minutes) with repeated measures over days. Fisher's Least Significant Difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

RESULTS

Acoustic Startle Responses

Analysis of variance of baseline startle amplitude failed to reveal significant differences among treatment conditions prior to the experimental treatment, $F<1$. Analysis of variance of startle amplitude associated with the treatment conditions revealed a significant main effect of Treatment, $F(9,31)=4.32$, $p<.01$ and a significant Treatment x Day interaction, $F(27,93) =1.68$, $p<.05$. Fisher's LSD multiple comparisons revealed that on the Immediate day startle scores among mice exposed to the predator odor (PO) were significantly elevated immediately, 30 minutes and 1 hour in comparison to the 2 or 4 hour intervals following odor presentation. Likewise, startle scores were significantly elevated 48 hours among mice tested immediately following predator odor exposure in comparison with either the 2 or 4 hour post-odor evaluation sessions. In contrast, there were no significant differences in startle scores among CO mice at any of the test intervals following odor exposure. Startle scores among mice assessed immediately (5 minutes) following odor presentation were elevated in PO mice in comparison to CO mice across all 4 test sessions (i.e. immediately, 24 hours, 48 hours and 168 hours). Startle scores among mice assessed 30 minutes following odor presentation were elevated in PO mice relative to CO mice across all 4 test sessions. Startle scores among mice

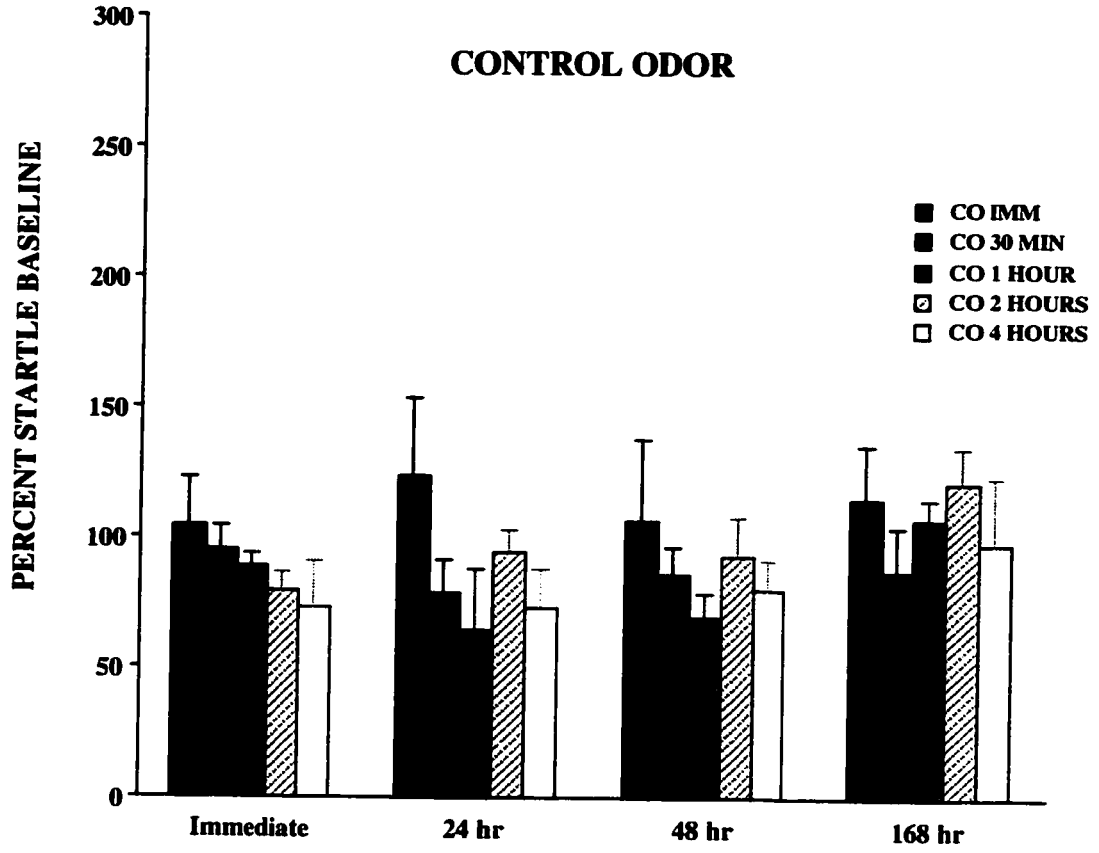
assessed 1 hour following odor presentation were elevated in PO mice in comparison to CO animals immediate, 24, and 48 hours but not 168 hours. Startle scores among mice assessed 2 hours following odor presentation were elevated in PO mice compared to CO mice 24 and 168 hours. There were no significant differences in startle scores among mice assessed 4 hours following odor presentation during any of the test sessions (see Figure 2A.1).

Insert Figure 2A.1 about here

Odor Exposure Duration

Analysis of variance of the baseline startle amplitude failed to reveal significant a priori differences among treatment conditions, $F<1$. Analysis of variance of the startle amplitude associated with odor presentation revealed a significant main effect of Treatment, $F(1,28)=30.47$, $p<.001$, a significant main effect of Time, $F(2,28)=5.27$, $p<.05$, a significant Day x Time interaction, $F(6, 84) =2.16$, $p<.05$ and a significant Day x Treatment x Time interaction, $F(6, 84) =2.70$, $p<.05$. Multiple comparisons analysis, employing Fisher's Test of Least Significant Difference of the simple simple main effects associated with the Day x Treatment x Time interaction, revealed significantly higher startle amplitudes among PO mice following a 10 minute exposure relative to either CO or HC mice at the immediate and 48 hour post-stressor intervals. Mice exposed to 5 minutes of PO exhibited enhanced startle amplitudes relative to CO mice at each of the post-stressor intervals examined. Mice exposed to 2 minutes of PO exhibited significantly enhanced startle amplitudes relative to CO mice only at the 168 hour post-stressor interval. Among PO mice, mice exposed for 5 minutes or

Figure 2A.1: Mean (± S.E.M.) startle amplitude (% of baseline startle scores) of mice following 10 minute exposure to a novel environment (CO) (top panel) or rat odor (PO) (bottom panel) tested immediately, 30 minutes, 1 hour, 2 hours or 4 hours as well as 24, 48 and 168 hours following odor presentation. Note: * depicts a statistically significant difference ($p < .05$) of CO and PO odor treatments at the identical post-stressor interval.


CONTROL ODOR
PERCENT STARTLE BASELINE
300
250
200
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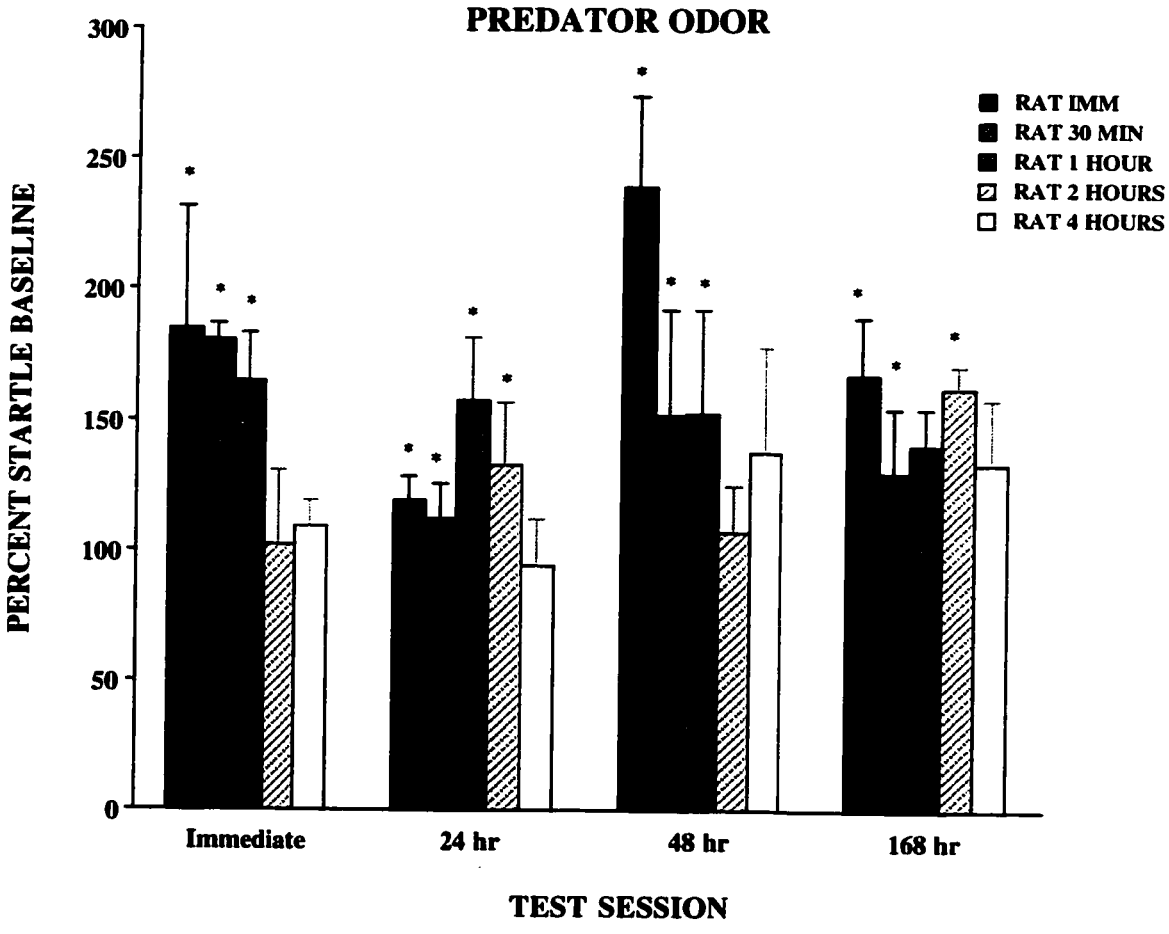
100
50
0
CO IMM
CO 30 MIN
CO 1 HOUR
CO 2 HOURS
CO 4 HOURS
Immediate
24 hr
48 hr
168 hr


PREDATOR ODOR
PERCENT STARTLE BASELINE
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Immediate
24 hr
48 hr
168 hr
TEST SESSION

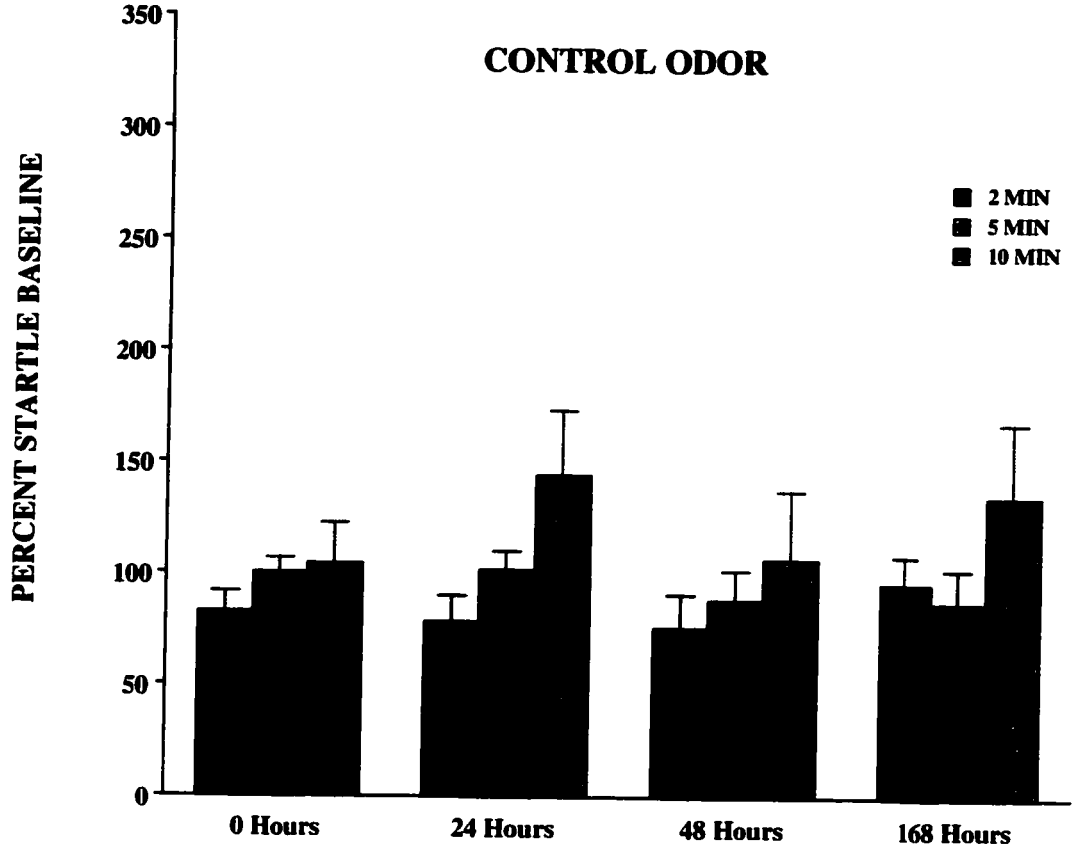
10 minutes exhibited increased ASR relative to mice exposed to 2 minutes of PO at the immediate, 24 hour and 48 hour post-stressor intervals. Among CO mice, a 2 minute, 5 minute or 10 minute exposure session did not result in variations in startle reactivity at any post-stressor interval examined. Mice in the CO condition did not differ from HC mice at any post-stressor interval examined (see Figure 2B.1).

Insert Figure 2B.1 about here

DISCUSSION

The data of Experiment 1 revealed that the mere exposure of CD-1 mice to the novel environment described by clean shavings was sufficient to induce anxiety in the light-dark box relative to home cage housed mice. In comparison, the behavioural profile of home caged housed mice in the light-dark box was consistent with previous reports of non-stressed mice in this apparatus under low illumination (60 watts cf. 40 watts) (Costall et al., 1989). In particular, home cage odor housed mice spent approximately 60-70% of the trial block in the illuminated chamber section. Exposure of mice to either the scent of a novel environment or the rat odor decreased dark compartment latency relative to home cage housed subjects. Decreased dark chamber latency and cumulative time spent in the light chamber of the light dark box was evident among mice of the clean shavings and rat odor conditions at all time intervals examined following odor presentations. The influence of predator odor on decreased illuminated chamber occupancy was exaggerated immediately following odor exposure relative to CO and HC mice. In contrast, decreased light chamber appeared among mice tested in the light-dark box 30 minutes following exposure to clean shavings relative to home-caged

Figure 2B.1: Mean (± S.E.M.) startle amplitude (% of baseline startle scores) of mice following a 2, 5 or 10 minute exposure to a novel environment (CO) (top panel) or rat odor (PO) (bottom panel) relative to home-caged (HC) mice, immediately, 24 hours, 48 hours and 168 hours following odor presentation. Note: * depicts a statistically significant difference ($p < .05$) of CO and PO odor treatments at the identical post-stressor interval following either a 2 minute, 5 minute or 10 minute odor presentation.


CONTROL ODOR
PERCENT STARTLE BASELINE
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24 Hours
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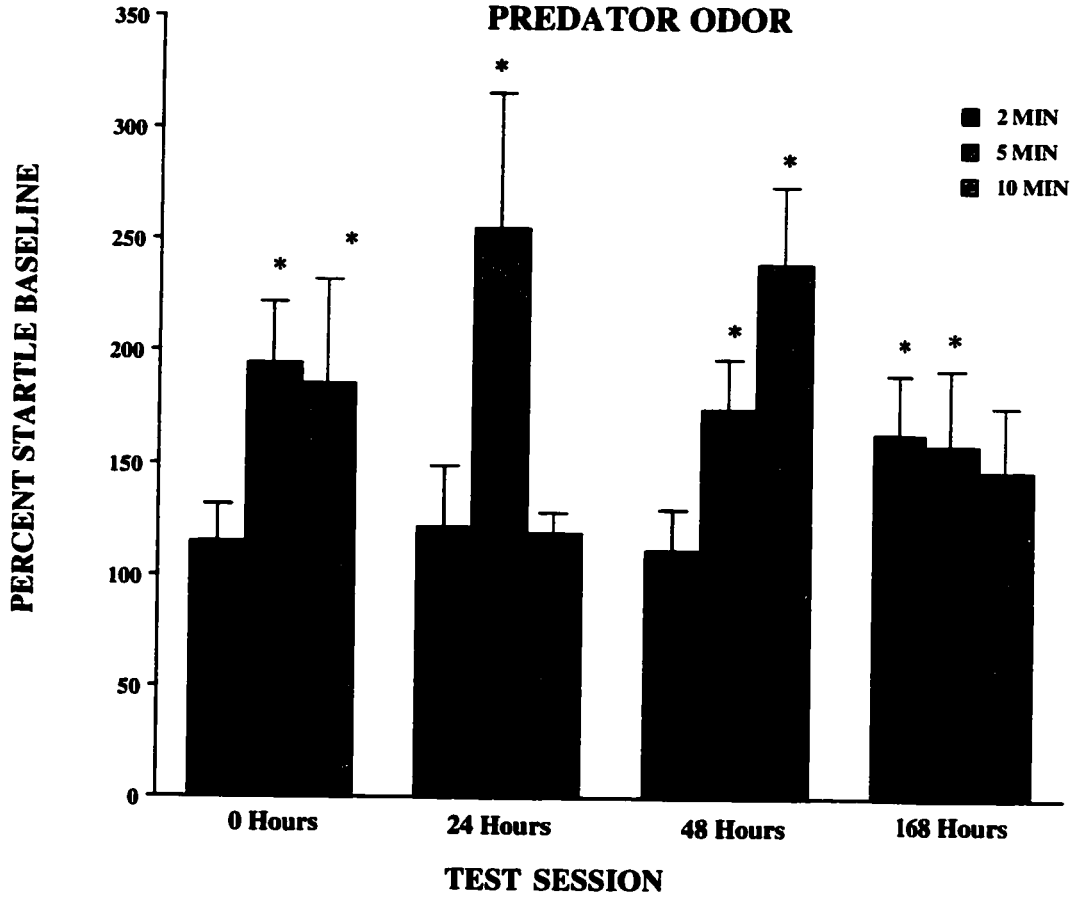
168 Hours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0 Hours
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168 Hours
TEST SESSION

animals. Interestingly, such a behavioural profile between these apparently disparate odor exposure groups was restricted to the initial 5 minute trial block of the light-dark box exercise. The demonstration of anxiety-like behavior in the light-dark box following exposure to clean shavings in a novel environment or predator odor are certainly consistent with investigations which detail mild stressor experience and exploratory propensity in rats (Miczek et al., 1999), motivational tendency and palatable food consumption (Di Chiara et al., 1999), light-dark box anxiety (MacNeil et al., 1997) as well as defensive response styles among rats and mice (Blanchard et al., 1997; Blanchard et al., 1990; Kemble & Bolwahnn, 1997) in resident-intruder, tail pinch, abbreviated footshock and predatory odor paradigms respectively.

It is intriguing that the influence of contextual variations associated with odor presentation is salient in Experiment 1. While the influence of rat odor encounter is certainly prominent in the light dark task, the impact of the clean shavings on light dark behavior among CD-1 mice is also conspicuous. It is noteworthy that the impact of this manipulation is reminiscent of the report of Tassin et al. (1980). In that investigation, mice exposed to an open field displayed behaviours which appeared to define anxiety (e.g., decreased locomotor activity/exploration). In the current application, mice appear to have a readily definable olfactory cue associated with the novel environment. To be sure, while the rat odor provided a putative threat associated with predation, the novel environment provided a scent which may have approximated the scent associated with the light-dark box. Finally, home cage housed animals were provided with a familiar scent and did not demonstrate anxiety in the light-dark box.

Consistent with the behavioural repercussions of neutral odor or predator odor encounter in a novel environment on light-dark box behaviour, mice in the respective

treatment conditions displayed augmented CCK mRNA levels in the BLA relative to home cage housed immediately following odor exposure. Interestingly, among mice exposed to clean shavings BLA CCK mRNA was enhanced relative to home cage housed animals, immediately, 1 hour and again at 4.5 hours following stressor exposure. Such an effect was absent among mice exposed to the predator odor. It is curious that the appearance of amygdaloid gene expression effected by a neutral odor encounter in a novel environment undergoes intervals of enhanced activation, followed by acquiescence and reactivation at ensuing time frames. Such an expression of neural activation in response to stressors was initially operationalized as phasic reaction of basolateral amygdaloid neurons to neurogenic stressor encounter (Davis et al., 1998). In contrast, medial amygdaloid nucleus CCK mRNA values were comparable among mice exposed to clean shavings or mice which encountered the rat odor. Nevertheless, MEA CCK mRNA among mice exposed to the neutral odor or the putative predator odor was enhanced relative to CCK expression in this identical nucleus among home cage housed mice. These data suggest that the medial amygdaloid nucleus fails to accord saliency to demonstrably disparate stressors. It should be noted that phasic activation of basolateral amygdaloid rather than medial amygdaloid neurons has been associated with presentation of novel or threatening stimuli in rats. Functional arguments available to date profer that such amygdaloid signals provide a neuronal preparedness to impending stressor encounter (Davis et al., 1998; Dielenberg et al., 2001). Indeed, such phasic neuronal responsivity is evident in both the basolateral and central amygdaloid nuclei following stressor imposition (e.g., footshock, predator odor). In this respect, CCK in the basolateral and central amygdaloid nuclei appears to participate in vigilance, memory and anticipatory responsivity among rats

in tasks which emphasize footshock avoidance and the Morris water task (Davis & Shi, 1999; Flood et al, 1992; Huston et al, 1998; Pitkanen et al, 1997; Voits et al, 2001).

In contrast to CCK mRNA alterations within the basolateral amygdaloid nucleus in response to clean shavings, CD-1 mice exposed to the identical odor in the novel environment demonstrated increased CCK mRNA in the mPFC and VTA immediately, 1.5 hours, 2.5 hours, and 4.5 hours following odor presentation and light dark box activity relative to home cage animals. Among CD-1 mice exposed to the odor of a rat, CCK mRNA in the mPFC and VTA were enhanced relative to home-caged animals. The increase in CCK mRNA appeared 1.5 hours, 2.5 hours and 4.5 hours following predator odor encounter and behavioral testing. Interestingly, the emergence of the demonstrable CCK mRNA increase in the VTA and mPFC among either the neutral or predator odor exposed mice was temporally and qualitatively variable. For example, while the mesolimbic responsivity of mice to the neutral odor was immediate, the neural response in these identical sites was delayed for 30 minutes among mice exposed to the odor of a rat. Yet, the neutral odor effected enhanced VTA and mPFC CCK mRNA responsivity for at least 3.5 hours following normalization of the mRNA activity. In contrast, increased predator odor CCK mRNA was attenuated for 1 hour, followed by a re-emergence of increased CCK mRNA activity for a 3 hour interval in comparison to the profile evident among home cage housed mice. Among mice exposed to rat odor, increased VTA and mPFC CCK mRNA levels were conspicuous 30 and 60 minutes following odor presentation relative to CO and HC mice. These data are interesting in view of the findings of Zangrossi & File (1992) who reported conspicuously elevated anxiety indices among rats in the social interaction and elevated plus maze paradigms 35-60 minutes following cat odor exposure. Taken together, exposure of CD-1 mice to either a novel environment with a distinctive

neutral odor or predator odor appears sufficient to provoke demonstrable behavioural indices of anxiety in the light dark task. In particular, the variable temporal expression of enhanced CCK activity in the BLA or VTA and mPFC suggests that CCK activity in the BLA may be involved in the initiation of anxiety-like behaviour while mesocortical CCK may sustain anxiety over longer periods of time following predator odor, in particular.

Cholecystokinin is colocalized with DA in the VTA and mesencephalic DA/CCK neurons project to the nucleus accumbens, central amygdaloid nucleus and mesocortex (Fallon et al, 1983; Hokfelt et al., 1980; Seroogy et al., 1989; Studler et al., 1981). Exposure of rats and mice to psychogenic (e.g., predator odors; social defeat) or neurogenic (e.g., footshock) stressors promotes the release of mesocorticolimbic DA (Cabib et al, 1988; Deutch et al, 1985; Imperato et al, 1989; Keefe et al, 1990; Saavedra, 1982; Watanabe, 1984) and CCK (Becker et al, 2001; Harro et al, 1996; Pavlasevic et al, 1993; Rosen et al. 1992; Siegel et al, 1987). In rats, increased mesocortical CCK-8S binding and extracellular levels of CCK are associated with increased levels of anxiety in the elevated plus maze (Harro et al., 1990). The basolateral amygdaloid nucleus is associated with the relay of emotionally laden stimuli from all sensory modalities to the central amygdaloid nucleus (Davis & Shi, 1999). It has been previously reported that while large numbers of CCK containing cell bodies are located in the basolateral and cortical nuclei of the amygdala, only a few labeled neurons were found in the central nucleus of the amygdala (Pu et al., 1994; Roberts et al., 1982). The basolateral nucleus of the amygdala plays a significant role in affective behaviour that is regulated by dopamine (DA) afferents from the mPFC. It has been demonstrated that activation of mPFC and VTA DA neurons attenuate BLA neuronal activity in vivo and in vitro via recruitment of BLA interneurons that suppress cortical inputs (Rosenkranz & Grace, 1999; 2001). Interestingly,

the application of mild stressors is associated with alterations in mesocortical DA and basolateral amygdaloid CCK. For example, social isolation in rats from weaning is associated with increased DA turnover in the basolateral amygdala, decreased DA turnover in the mPFC (Heidbreder et al, 2000) as well as increased CCK mRNA in the basolateral amygdala, prefrontal cortex and VTA relative to group housed animals (Del Bel & Guimaraes, 1997). It will be recalled that Pavlasevic et al. (1993) demonstrated that while predator associated olfactory cues increased CCK-4 concentrations in the olfactory bulb, frontal and central cortices, dorsal striatum, nucleus accumbens, central amygdaloid nucleus and the nucleus of the solitary tract exposure of a rat to the smell of a cat also increased cortical DA release. In addition, a 20 minute exposure of rats to fox odor increased DA metabolism within the mPFC and BLA (Morrow et al., 2000). Taken together, the distinct temporal expression of CCK mRNA from the basolateral amygdala following exposure of CD-1 mice to a novel environment or predator odor may follow from mesocortical DA activation following stressor imposition. Consistent with such an analysis, mild stressor imposition, including neutral scent applications, elicits anxiety in the light-dark box among infrahuman subjects occasioned by perturbations of CCK expression in mesolimbic DA sites, including the basolateral amygdala, VTA and mesocortex.

The behavioural data secured in Experiment 2 clearly reveal that exposure of CD-1 mice to rat odor enhanced acoustic startle (ASR) for up to one week following odor presentation. It should be underscored that exposure of mice to clean shavings failed to enhance ASR relative to HC mice. The pattern in which predator-evoked anxiety was expressed appears to mirror the duration of exposure to predator odor as well as the length of time following odor exposure that behavioural testing occurred. For example, the data

indicated that a 5-minute exposure of mice to predator odor enhanced the ASR at each of the post-stressor intervals examined for at least 168 hours. However, such a response was not observed if mice were exposed for 2 minutes or 10 minutes. Indeed, a 10 minute exposure of mice to predator odor produced elevations in the ASR immediately and 48 hours after exposure but not at the 24 hour or 168 hour test interval. However, if the startle stimulus was withheld 30 minutes or 1 hour following rat odor exposure, CD-1 mice exhibited enhanced startle at all the post-stressor interval comparable to a 5 minute exposure. When the startle stimulus was withheld for 2 hours following odor exposure PO mice exhibited enhanced startle only at the 168 hour post-stressor interval. Finally, when the startle stimulus was withheld for 4 hours following odor exposure on the immediate test day, PO mice failed to exhibit elevated startle amplitudes at any of the test sessions employed. In contrast, the mere 2 minute exposure to predator odor enhanced ASR only at the 168 hour test session. Taken together, these findings suggest that the duration of soiled rat shaving exposure and/or the temporal association of the psychogenic stressor experience with the startle stimulus influences the expression of anxiety in CD-1 mice following stressor encounter.

It should be considered that the duration of mild psychogenic stressors influences mesolimbic neurochemical and peptide variations among rats and mice. It should be emphasized that the neurochemical variants associated with increased acoustic startle following rat odor exposure in the present study are unavailable. However, examination of relevant investigations which assess the influence of psychogenic stressor applications on behaviour and mesolimbic DA/CCK as well as employ pharmacological manipulations to influence behaviour following stressor imposition are appropriate. For example, a 10 minute

exposure of rats to the visual and olfactory cues associated with footshocked conspecifics enhanced VTA DA turnover, while a 30 minute exposure enhanced mPFC DA turnover (Kaneyuki et al., 1991). In a similar vein, 2 discrete handling episodes over a 20 minute period increased mPFC extracellular DA 120%, while 20 minutes of continuous handling increased mPFC extracellular DA to greater than 200% (Feenstra et al., 1995). Finally, a 20 minute exposure of rats to a novel environment increased mPFC DA release 120% (Feenstra et al., 1995) while a 30-minute exposure increased DA release in the mPFC and nucleus accumbens 130% of baseline values (Tidey & Miczek, 1996). In defeated intruders, extracellular dopamine levels in accumbens and prefrontal cortex were increased further (approximately 160% of baseline) (Tidey & Miczek, 1996). Moreover, the expectation of social defeat increased anxiety-like behaviour and CCK levels in the rat cortex. Pretreatment with diazepam prevented the behavioural expression of anxiety and increased CCK levels (Becker et al., 2001). Although the behavioural alterations associated with these psychogenic stressor applications are limited, it is likely that site specific neurochemical alterations incite variations in stressor-associated behavioural profiles. Indeed, the severity of the stressor experience may be quantitatively defined. For example, a 2 minute-predator exposure is mildly stressful compared to a predator encounter of 10 minutes. Unfortunately, there is a relative paucity of information pertaining to the effects of duration of psychogenic stressor encounters and CCK release (c.f., Siegel et al., 1984 and 1987). Taken together, it should be considered that 2, 5 or 10 minutes of predator exposure may induce a stressor severity gradient of DA and CCK release from selective mesolimbic sites. These site specific neurochemical alterations may be associated with the expression of specific ASR patterns, as well as light-dark box anxiety, among CD-1 mice following acute predator odor exposure.

In the present investigation, a 5-minute exposure of mice to the smell of a rat elevated ASR at each of the post-stressor intervals examined. Previous investigations have revealed that systemic or intra-basolateral amygdaloid nucleus administration of NMDA receptor antagonists, MK-301, AP7 or CPP prior to but not following predator odor exposure prevented the expression of anxiety in rats in the elevated plus maze and acoustic startle paradigms 1 week following an initial 5 minute predator exposure session (Adamec et al., 1999; Adamec et al., 1997, respectively). These data suggest that NMDA receptors are involved in initiation of neural changes (i.e., long-term potentiation) mediating lasting increases in anxiety following an acute psychogenic stressor exposure. In contrast, systemic administration of the CCK_B antagonist L-365, 260, 30 minutes prior or 30 minutes following cat exposure, prevented the expression of anxiety in rats in the elevated plus maze 1 week following the initial 5 minute predator exposure session (Adamec et al, 1999; Adamec et al, 1997, respectively). In particular, the protracted effects of predator stress on anxiety-like behaviour in the ASR likely involve initiation of long-term potentiation like effects (LTP) by NMDA receptors within the basolateral nucleus and persistence of LTP by CCK_B receptor activation within other central sites involved in the startle response, including the central amygdaloid nucleus and caudal pontine reticular nucleus.

While the 5-minute exposure of CD-1 mice to predator odor produced elevations in the ASR at each post-stressor interval examined, the 2-minute exposure to soiled rat bedding failed to produce elevations in the ASR until 168 hours post-stressor. The delayed onset of enhanced ASR likely reflected a period of latency between stressor encounter and production of anxiety-like behaviour. A similar phenomenon has been reported in war veterans, survivors of motor vehicle accidents and natural disasters, as well as abused women, who

develop symptoms of post-traumatic stress disorder 6 months following traumatic experiences (Buckley et al., 1996; Lim, 1991; Morgan et al., 1997; Ramchandani, 1990; Solomon et al., 1995). In addition, startle stimuli themselves are aversive (Borszcz et al., 1989) and may induce protracted states of fear or anxiety among PO mice. Taken together, repeated exposure of CD-1 mice to the startle stimulus following predator exposure may increase behavioural and neurochemical responses resulting in a delayed exaggeration of startle reactivity. Such phenomenon underlies behavioural sensitization (Stam et al., 2000).

The 10 minute exposure of CD-1 mice to predator odor produced an oscillating pattern of increased startle amplitude on the immediate and 48 hour test session when the startle stimulus was presented immediately following odor presentation. It has been previously demonstrated at high fear levels, imposed with more severe stressors, the amygdala enhances dorsolateral periaqueductal gray neuronal activity, which in turn acts in an inhibitory fashion to attenuate startle (Walker et al., 1997). In particular, dorsolateral periaqueductal gray CCK neuronal activation induces defensive behaviours, including fight and flight, which are incompatible with the expression of startle (Mongeau & Marsden, 1997). However, when the startle stimulus was withheld 30 or 60 minutes following odor presentation mice exhibited enhanced startle at all the post-stressor intervals similar to the 5-minute exposure. These data suggest an incubation period that facilitates LTP-dependent processes underlying the expression of behavioural change and various mesolimbic sites may interplay to produce temporally dependent expression of startle under varying stressor intensities.

In summary, it is likely that novel odors and predator odors, promote increased CCK availability in the central and basolateral amygdala associated with increased vigilance and

protracted indices of fear and startle reactivity. In contrast, increased CCK in the VTA and mesocortex may be associated with gradient alterations in motivation and anxiety and concomitant reductions in exploratory tendencies. Consideration of increased CCK availability in specific central sites associated with anxiety symptoms, for example, is intriguing in view of the wealth of data relating sustained elevation of central CCK to the protracted impact of stressors on motivation and anxiety in clinical samples. In the present series of investigations, the mere exposure of mice to clean shavings was sufficient to induce anxiety in the light dark box while this identical stressor manipulation was without effect in provoking enhanced startle. It will be recalled that mild stressors, including acute elevated plus maze exposure (i.e., novel environment) effectively induce alterations in mesocortical CCK while fail to induce alterations of amygdaloid CCK. In effect, the neural mechanisms that subserve anxiety in the light-dark box are not congruent with those that underlie expression of acoustic startle. To be sure, CCK activity within sub-nuclei of the startle circuit would need to be evaluated following exposure of CD-1 mice to clean shavings or predator odor. Although, such a CCK based explanation is speculative, it is certainly one deserving of consideration. Taken together, these data suggest that salient stressors elicit site-specific CCK alterations which contribute to specific behavioural alterations in animals reminiscent of the clinical condition.

EXPERIMENT 3

It is well documented that stressful life events contribute to the etiology of mood disorders (Post et al., 1998). A role for CCK in the induction of anxiety in infrahuman subjects (Cohen et al, 1998), promotion of panic in humans (Bradwejn et al, 1990) and co-expression of depression (Lofberg et al, 1998) appears to be conspicuous. Several

investigators have argued that some species specific stressors may be relevant in animal models of affective disorders (Blanchard & Blanchard, 1988). Indeed, exposure of rats and mice to predator odor elicits neurochemical and behavioural perturbations which include increased anxiety-like behaviour in the elevated plus maze (McGregor & Dielenberg, 1999) and light-dark box (Adamec, 2001), increased freezing in novel environments (Hotsenspiller & Williams, 1997), increased risk assessment (Blanchard et al., 1998), decreased sucrose consumption (Calvo-Torrent et al., 1999), increased concentrations of CCK-4 in the olfactory bulb, mesocortex, nucleus accumbens and central amygdaloid nucleus and increased CCK-8S concentrations in the striatum (Pavlavesic, 1993). In particular, mere presentation of predator cues is sufficient to incite central mesolimbic CCK variations as well as behaviours associated with mild anxiety states.

The application of odor substances extracted from the scent glands of the fox and the weasel favors quantitative evaluation of experience intensity while allowing comparison of non-predatory odor intensity (see Lu et al., 1993; Zibrowski et al., 1998). For example, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) and 2-propythietane (PT) are agents isolated from the fox scent gland (vulpes vulpes) (Vernet-Maury, 1980) and weasel (mustela erminea) (Crump & Moors, 1985), respectively. TMT induces comparable levels of fear in rats relative to whole fox feces exposure as revealed by suppressed open-field activity and elevated corticosterone levels (Morrow et al., 2000; Vernet-Maury et al., 1980; Vernet-Maury et al., 1992). It is suspected that these effects are accompanied by protracted risk assessment behaviours thus providing a particularly relevant threat and potent fear inducing stimulus. The present experiment assessed the immediate defensive behavioural repertoire, as well as the expression of the preproCCK mRNA from the VTA, medial prefrontal cortex and basolateral

nucleus of the amygdala, among CD-1 mice acutely exposed to TMT, butyric acid or saline as well as the immediate behavioural consequences of such substance exposure in the light-dark box.

MATERIALS AND METHODS

Subjects and Odor Apparatus

Forty eight, naive, 3 month old male, CD-1, mice (n=48) were exposed to either 2,5-Dihydro-2, 4,5-trimethylthiazoline (TMT) the major component of the anal gland secretions of the red fox purchased from Phero-Tech, (Delta, B.C.), butyric acid (Sigma), a pungent, non-predatory, control odor or physiological saline, the odor free control substance. All odorants were impregnated on sterile gauze (5 x 5 cm) and placed in the home cage in 10 cm glass petri dishes covered by wire mesh. A Canon ES950 video camera mounted above the cage recorded the behaviour of mice during odor presentation.

Behavioural Testing

Light-Dark Apparatus

The light-dark apparatus was previously described in Experiment 1.

Procedure

Mice were initially habituated to placement beneath the video camera with the glass petri dish placed in their home cage but no odors present for 10 minutes on three consecutive days. On each habituation trial, the wire cage top was removed and replaced with a clear Plexiglas sheet to enable observation of risk assessment behaviours. Mice were transported to another testing area where light-dark box performance would be recorded. Mice were not exposed to the light-dark box during the habituation procedure. Mice were gently retrieved

from the home-cage and handled briefly (2 minutes). Each animal was immediately returned to the home cage and transported back to main animal housing unit.

Following habituation procedures, mice were randomly assigned to predator (n=16), butyric acid (n=16), or saline treatment conditions (n=16). Based on previously established criteria employed in ensuring equal odor potency of these compounds were presented to each subject (Hotsenpiller & Williams, 1997) 35 µl of predator odor, 105 µl butyric acid or 105 µl sterile saline was applied to the sterile gauze pads and placed in the cage for 10 minutes. Twenty-four mice were then transported immediately (5 minutes) to the second experimental room where activity in the light-dark box was recorded (n=8 for each of the 3 odor groups). The remaining 24 mice did not experience light-dark box exposure and served as controls for apparatus exposure. Five animals from each odor and light-dark box group (N=30) were perfused intracardially with 4% paraformaldehyde (3.8% Sodium Borate) and brains removed for preproCCK mRNA in situ hybridization.

In Situ Hybridization: CCK mRNA Histochemistry

The complete protocol for CCK mRNA histochemistry was detailed in Experiment 1.

Data Analysis

Odor Presentation

The frequency of contacts with the odorant cloth, defensive burying, freezing, rearing, stretch attend postures and grooming as well as the duration of contact with the odorant cloth, defensive burying, and freezing behaviours among mice during odor exposure in the home-cage were scored. Briefly, complete cessation of movement except respiratory muscles (freezing), orientation toward the odorant cloth with the top of the back lower than the ears

(stretched attention), moving toward the odorant cloth while pushing or spraying bedding material towards it (defensive burying) or physical contact with the cloth (cloth contacts) were included as risk assessment behaviours of mice during odor presentation. Three non-agonistic behaviours (rearing, digging and grooming) were also scored in response to the odorant treatments among CD-1 mice in the home-cage. All behavioural scores were analyzed by one-way analyses of variance for independent groups following stressor treatment. Fisher's least significant difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

Light-Dark Paradigm

All behavioural scores were analyzed as described in Experiment 1 with the exception of the addition of reentry latency of mice into the white section following initial dark compartment entry.

Quantitative Analysis of CCK mRNA

The procedure for identification of CCK positive neurons was detailed in Experiment 1. The density of the respectively identified cell types (i.e., positively stained cells) were evaluated in the basolateral amygdaloid nucleus (BLA), ventral tegmental area (VTA), and mesocortex (mPFC) and were subjected independently to ANOVA as a 3 (Saline, Butyric Acid, Predator) x 2 (Light-Dark Box, No Light-Dark Box) design. Fisher's Least Significant Difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

RESULTS

Home Cage Exposure

Analysis of variance of the number of contacts with the odorant cloth [$F(2, 45)=42.47$, $p<.001$], freezing behaviour frequency [$F(2,45)=21.89$, $p<.001$], frequency of defensive burying behaviours [$F(2,45) =15.12$, $p<.001$] and stretch attend frequency [$F(2,45)=9.30$, $p.<.001$] among mice during odor presentation in the home cage revealed a significant main effect of odor. Mice exposed to butyric acid or predator odor contacted the odorant cloth less than mice exposed to saline while mice exposed to fox odor made significantly fewer contacts with the odorant cloth than mice exposed to either saline or butyric acid. Mice exposed to fox odor displayed significantly elevated instances of freezing relative to mice exposed to either saline or butyric acid while there were no significant difference in the frequency of freezing behaviour among mice exposed to saline or butyric acid. Mice exposed to butyric acid or predator odor displayed significantly elevated instances of defensive burying relative to mice exposed to saline while mice exposed to fox odor displayed significantly more incidents of defensive burying activity relative to mice exposed to saline or butyric acid. Finally, mice exposed to butyric acid or predator odor displayed a significant increase in the frequency of stretch attend behaviours in comparison to mice which were merely exposed to saline. There was no significant difference in stretch attend frequency among mice exposed to fox or butyric acid (See Figure 3.1).

Insert Figure 3.1 about here

Analysis of variance of rearing frequency among mice during odor presentation in the home cage revealed a significant main effect of odor, $F(2,45)=11.93$, $p<.001$. Mice exposed to butyric acid or predator odor displayed decreased rearing behaviour in comparison with mice exposed to saline. Mice exposed to predator odor during home-cage analysis displayed reduced rearing relative to mice exposed to butyric acid (See Figure 3.2).

Insert Figure 3.2 about here

Analysis of variance of the duration of (a) contact with the odorant cloth [$F(2,45)=24.72$, $p<.001$], (b) freezing [$F(2, 45)=9.45$, $p<.001$] and (c) defensive burying [$F(2, 45)=12.53$, $p<.001$] among mice during odor presentation in the home-cage revealed a main effect of odor. Mice exposed to butyric acid or predator odor displayed decreased duration of contact with the odorant cloth relative to mice exposed to saline while mice exposed to fox odor displayed decreased duration of contact with the odorant cloth relative to mice exposed to butyric acid. Mice exposed to fox odor displayed a longer duration of freezing relative to mice exposed to saline or butyric acid. There was no significant difference in the duration of freezing behaviour among mice exposed to saline or butyric acid. Finally, mice exposed to TMT displayed enhanced duration of defensive burying relative to saline or butyric acid exposed mice (See Figure 3.3).

Figure 3.1: Home cage behavioral frequency (± S.E.M.) exhibited among mice during a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treated mice while ** depicts a statistically significant difference ($p < .05$) between butyric acid and TMT odor treatments for a particular home-cage behavior observed during odor presentation.

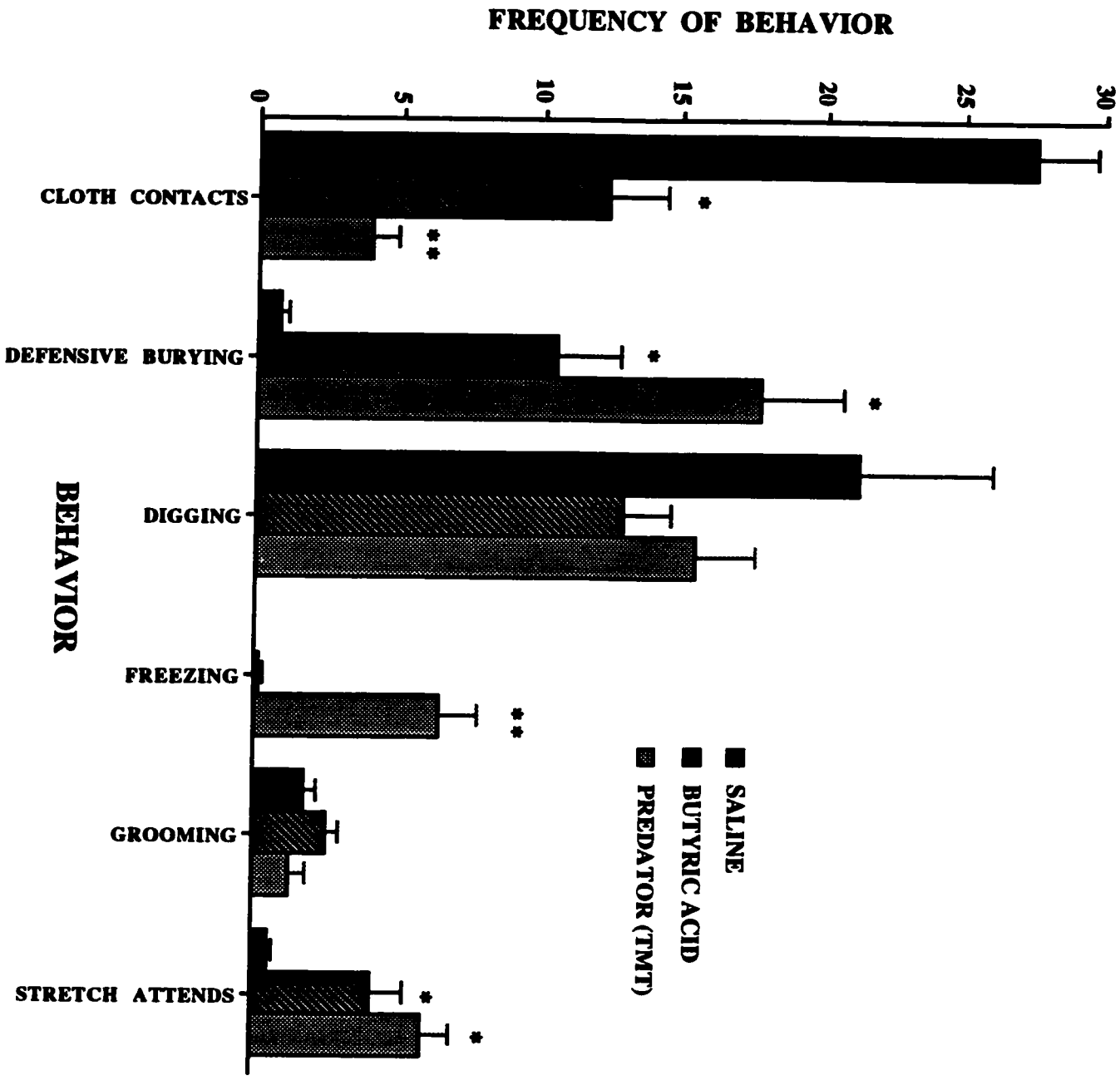
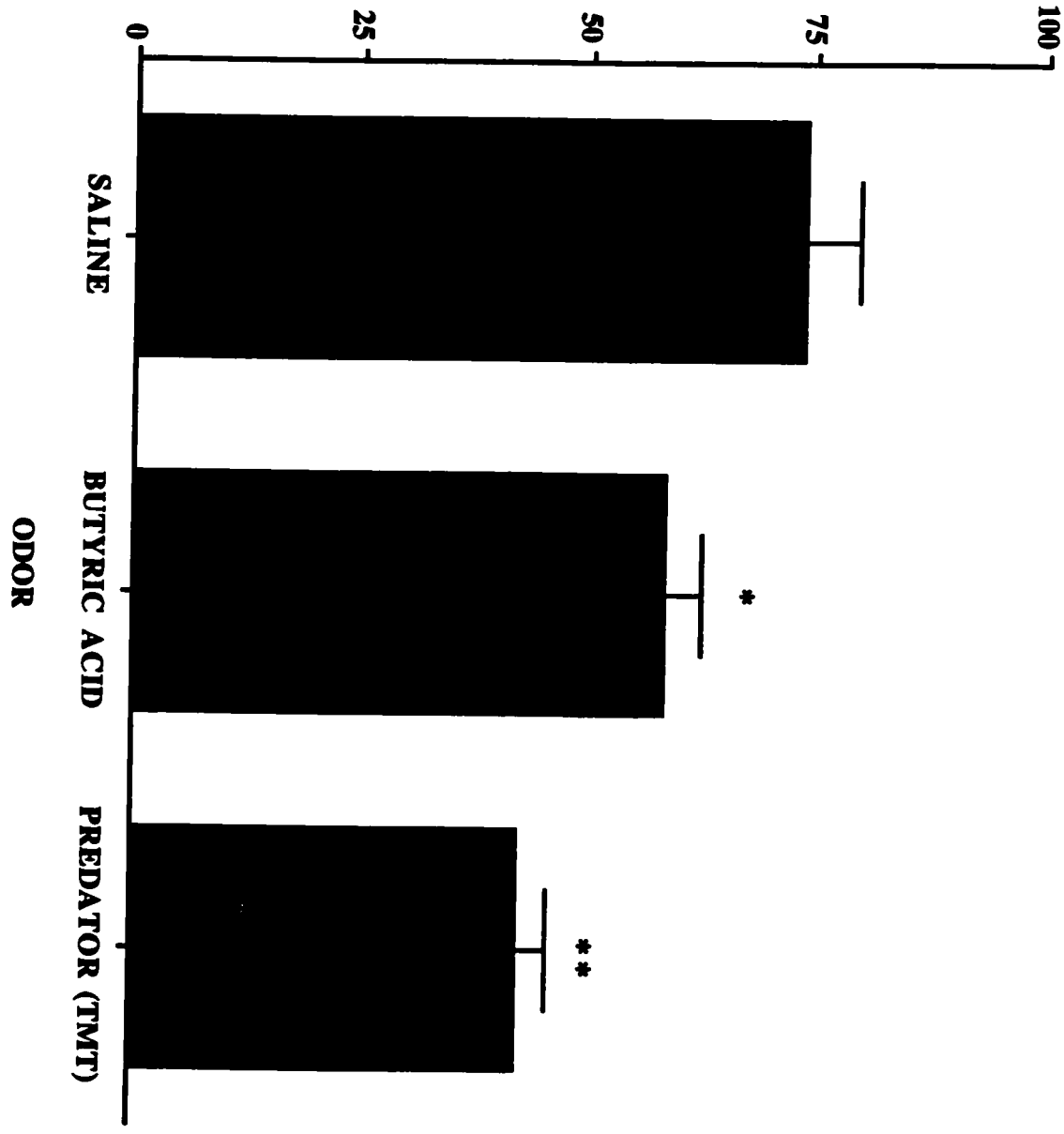

FREQUENCY OF BEHAVIOR
0
5
10
15
20
25
30
CLOTH CONTACTS
DEFENSIVE BURYING
DIGGING
FREEZING
GROOMING
STRETCH ATTENDS
BEHAVIOR
SALINE
BUTYRIC ACID
PREDATOR (TMT)

Figure 3.2: Home cage rearing frequency (± S.E.M.) exhibited among mice during 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treated mice while ** depicts statistically significant difference ($p < .05$) between butyric acid and TMT odor treatments.


FREQUENCY OF REARING
0
25
50
75
100
SALINE
BUTYRIC ACID
PREDATOR (TMT)
ODOR
*
**

Insert Figure 3.3 about here

Light-Dark Paradigm

Analysis of variance of the time spent light during the first and second 5 minute interval among mice immediately following odor exposure revealed a main effect of odor, $F(2, 21)=6.57$, $p<.01$ and $F(2,21)=6.80$, $p<.01$, respectively. During the first 5 minute segment of light dark box behavioural analysis, mice exposed to either butyric acid or predator odor spent less time in the light section of the apparatus relative to mice exposed to saline. During the second 5 minute segment of light dark box behavioural analysis, mice exposed to either butyric acid or predator odor spent less time in the light section of the apparatus relative to mice exposed to saline. Mice exposed to predator odor also spent less time in the lit portion of the apparatus relative to mice exposed to either saline or butyric acid (See Figure 3.4).

Insert Figure 3.4 about here

Analysis of variance of the cumulative time spent in the light chamber of the light dark box among mice immediately following odor exposure revealed a significant effect of odor, $F(2, 21)=7.63$, $p<.01$. Mice exposed to either butyric acid or predator odor spent less time in the light section of the apparatus relevant to mice exposed to saline (See Figure 3.5).

Insert Figure 3.5 about here

Figure 3.3: Duration of home-cage defensive behaviors (± S.E.M.) exhibited among mice during a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treatment while ** depicts a statistically significant difference ($p < .05$) between butyric acid and TMT odor treatments for a specific home-cage behavior observed during odor presentation.

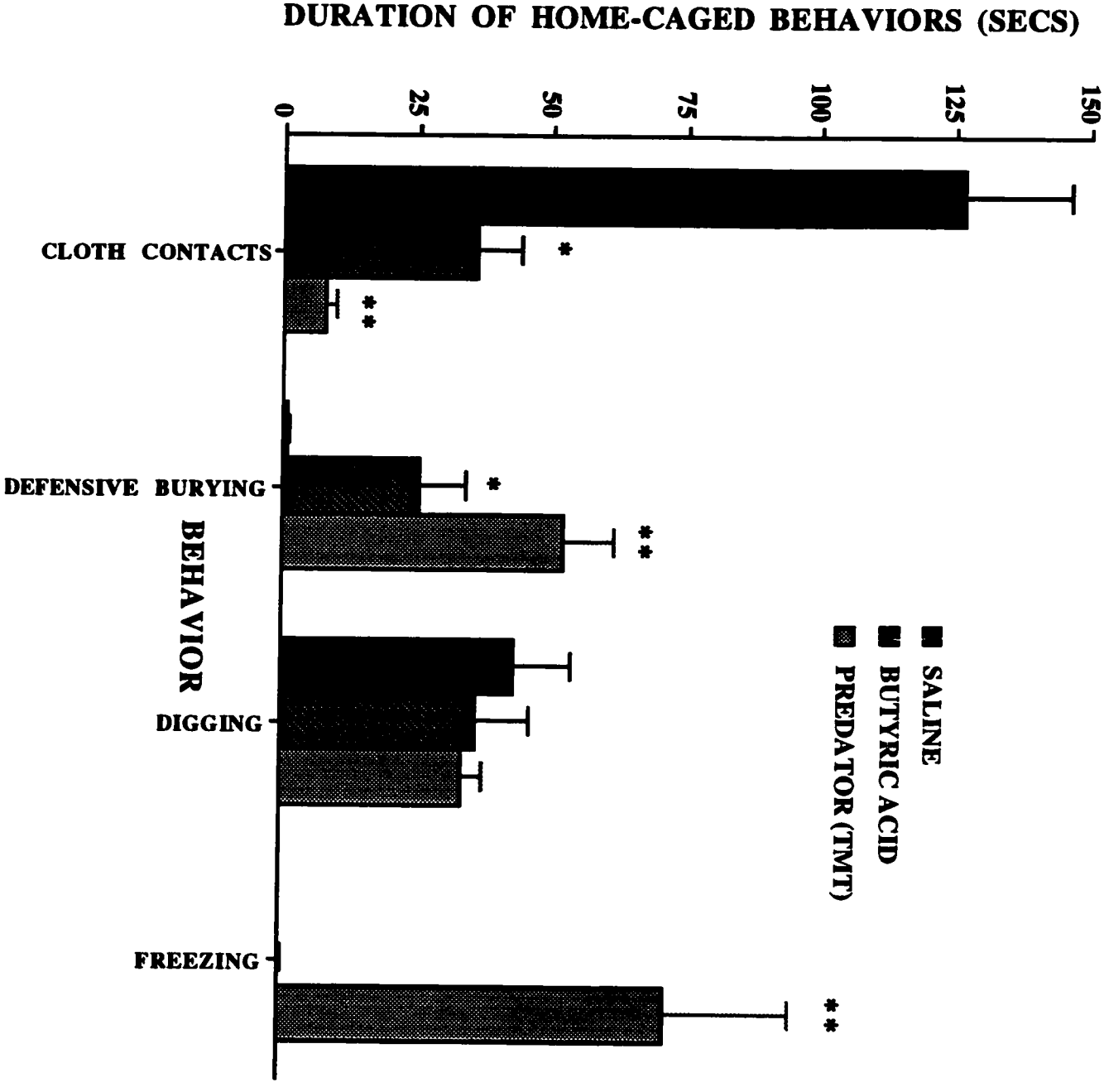

DURATION OF HOME-CAGED BEHAVIORS (SECS)
0
25
50
75
100
125
150
CLOTH CONTACTS
DEFENSIVE BURYING
DIGGING
FREEZING
BEHAVIOR
SALINE
BUTYRIC ACID
PREDATOR (TMT)
*
**
*
**
**

Figure 3.4: Mean (± S.E.M.) time in light during the first and second consecutive 5 minute intervals among mice following a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treatment while ** depicts a statistically significant difference ($p < .05$) of butyric acid and TMT odor treatments at the identical post-stressor interval.

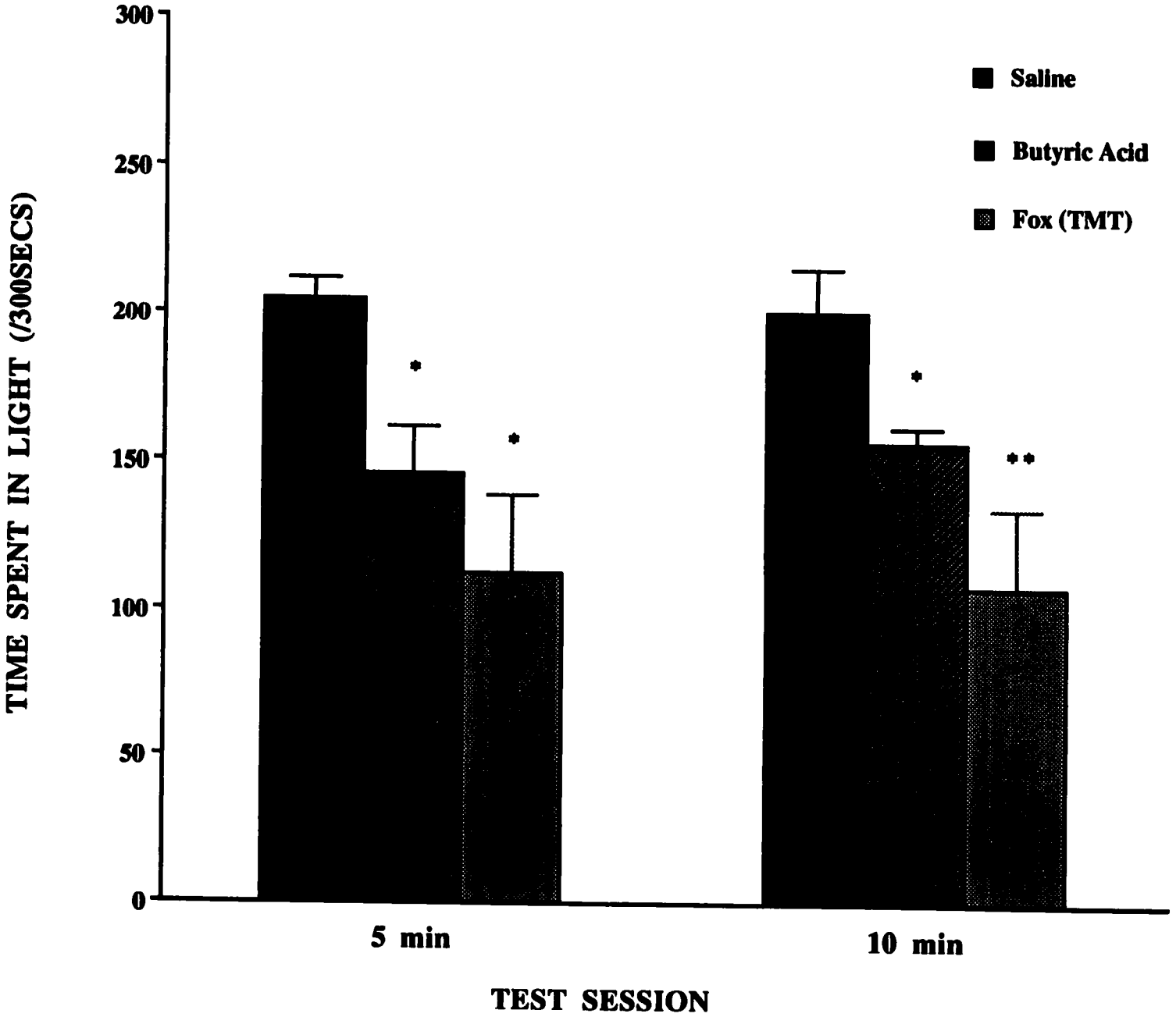
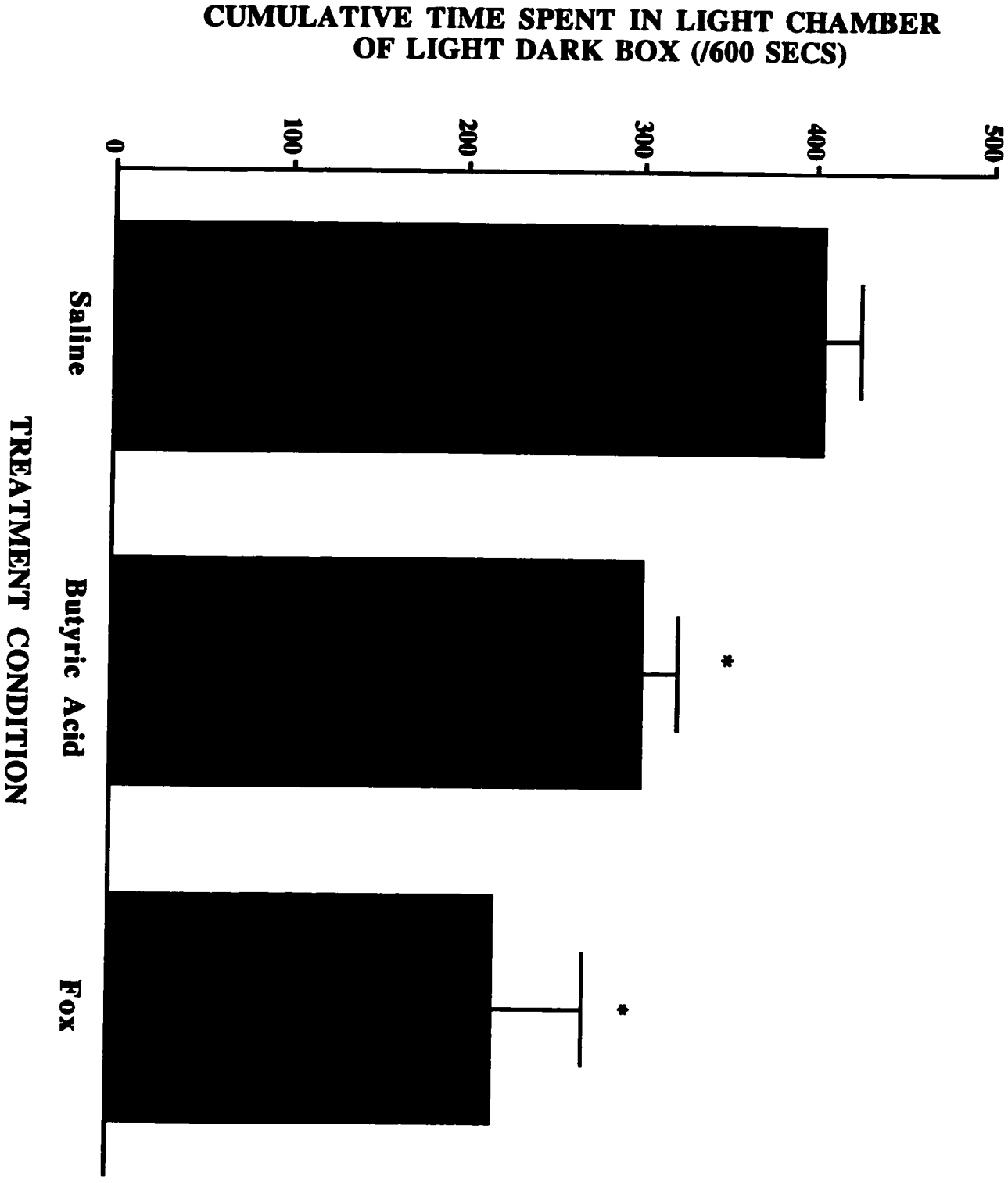

300
250
200
150
100
50
0
TIME SPENT IN LIGHT (/300SECS)
Saline
Butyric Acid
Fox (TMT)
*
*
*
**
5 min
10 min
TEST SESSION

Figure 3.5: Mean (± S.E.M.) cumulative time spent in the light portion of the light dark box among mice following a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treatment.


CUMULATIVE TIME SPENT IN LIGHT CHAMBER
OF LIGHT DARK BOX (/600 SECS)
0
100
200
300
400
500
Saline
Butyric Acid
Fox
*
*
TREATMENT CONDITION

Analysis of variance of light chamber reentry latency among mice immediately following odor exposure revealed a significant effect of odor $F(2, 21) = 3.87$, $p < .05$. Mice exposed to either butyric acid or predator odor took longer to reenter the light section of the apparatus relative to mice exposed to saline. There were no differences in reentry latency among mice treated with predator odor or butyric acid (See Figure 3.6).

Insert Figure 3.6 about here

Analysis of variance of cumulative light and dark compartment transitions of mice revealed a significant effect of odor $F(2, 21) = 3.66$, $p < .05$. Mice exposed to predator odor displayed decreased light and dark compartment transitions relative to mice exposed to butyric acid or saline. There were no differences in cumulative transitions among mice treated with saline or butyric acid (See Figure 3.7).

Insert Figure 3.7 about here

Quantitative Analysis of CCK mRNA

Basolateral Amygdala (BLA):

Analysis of variance of the optical density of CCK mRNA from the BLA among mice following odor presentation and light-dark box testing revealed an odor x light-dark box interaction $F(2,20) = 3.618$, $p < .05$. Exposure to the light-dark box increased CCK mRNA levels among mice previously exposed to butyric acid or predator odor relative to saline

Figure 3.6: Light chamber reentry latency (± S.E.M.) of mice following initial dark compartment escape immediately following a 10 minute saline. butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treatment.

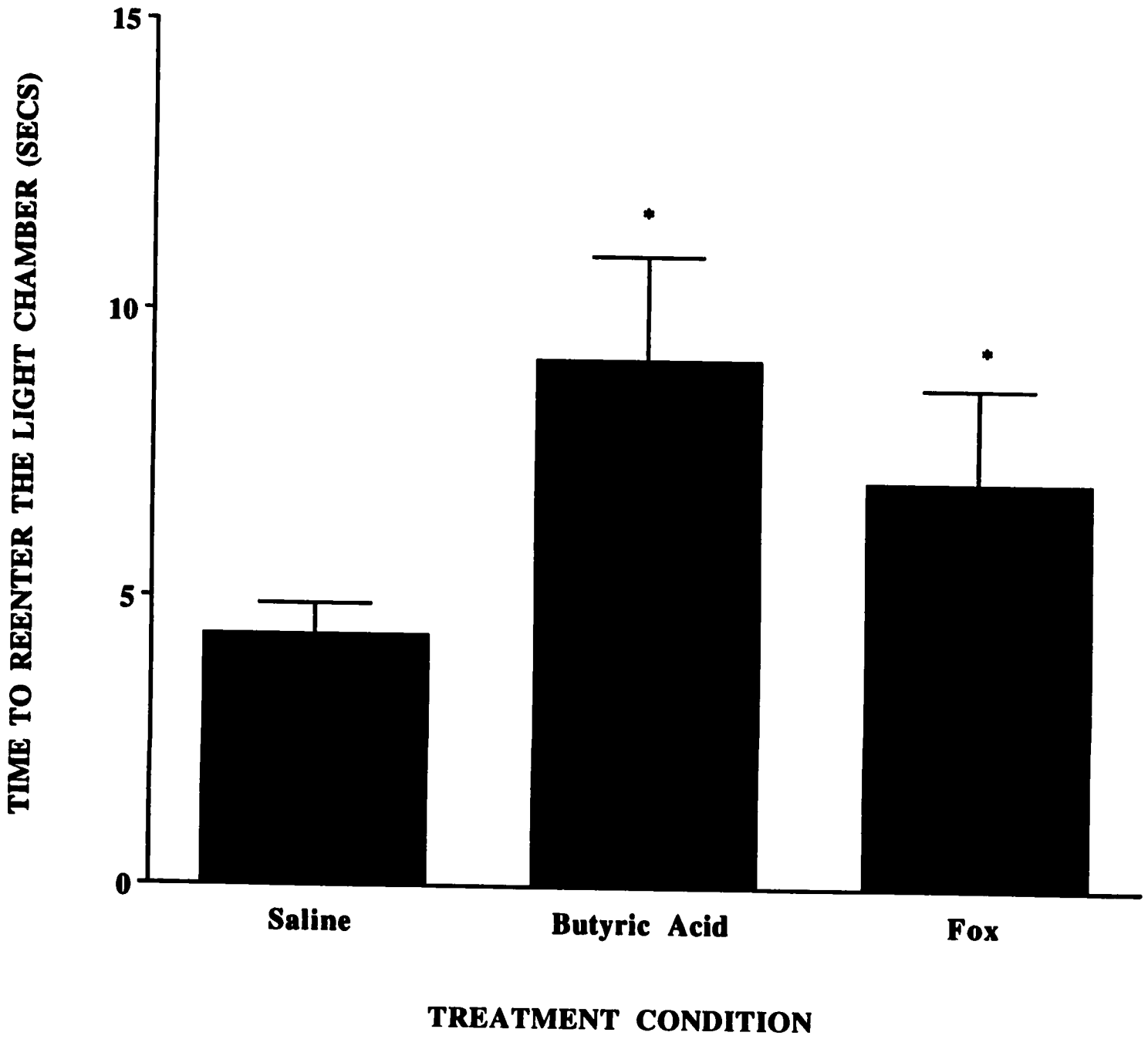
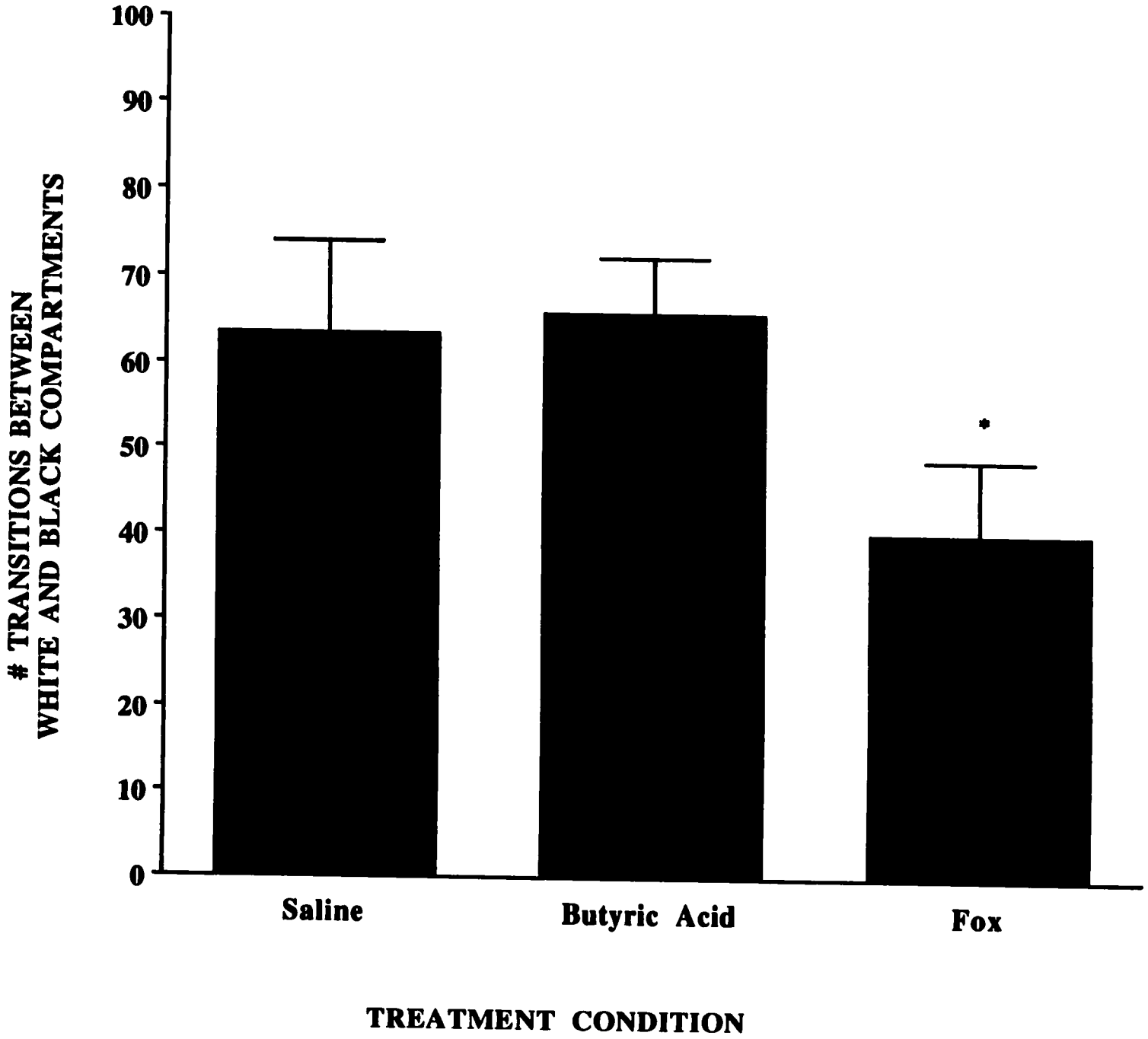

TIME TO REENTER THE LIGHT CHAMBER (SECS)
15
10
5
0
*
*
Saline
Butyric Acid
Fox
TREATMENT CONDITION

Figure 3.7: Cumulative (± S.E.M.) light and dark compartment transitions of mice immediately following a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treatment.


100
90
80
70
60
50
40
30
20
10
0
TRANSITIONS BETWEEN WHITE AND BLACK COMPARTMENTS
*
Saline
Butyric Acid
Fox
TREATMENT CONDITION

treated mice. There were no differences in CCK mRNA levels among odorant conditions in the No Light-Dark Box treatment condition (See Figure 3.8).

Insert Figure 3.8 about here

Medial Prefrontal Cortex (mPFC):

Analysis of variance of the optical density of CCK mRNA from the mPFC among mice following odor presentation and light-dark box testing revealed a main effect of odor $F(2,19) = 6.269$, $p < .01$ and a main effect of light-dark box exposure $F(1,19) = 4.456$, $p < .05$. Mice exposed to butyric acid or predator odor displayed elevated CCK mRNA relative to saline treated mice. Mice exposed to the light-dark box exhibited decreased CCK mRNA in the mPFC relative to mice not tested in the light-dark box (See Figure 3.9).

Insert Figure 3.9 about here

Ventral Tegmental Area

Analysis of variance of the integrated density of CCK mRNA from the VTA among mice following odor presentation and light-dark box testing revealed a main effect of odor $F(2,20) = 4.442$, $p < .05$. CCK mRNA was increased in mice exposed to butyric acid relative to saline or predator odor treated mice (See Figure 3.10).

Insert Figure 3.10 about here

Figure 3.8: Cell mean (± S.E.M.) and representative photomicrographs for optical density of BLA CCK mRNA following a 10 minute saline (SA), butyric acid (BA) or fox odor (TMT) presentation among mice in light-dark and no light-dark testing schedules. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treatment among mice in the light-dark box condition.

Basolateral amygdala
CCK mRNA

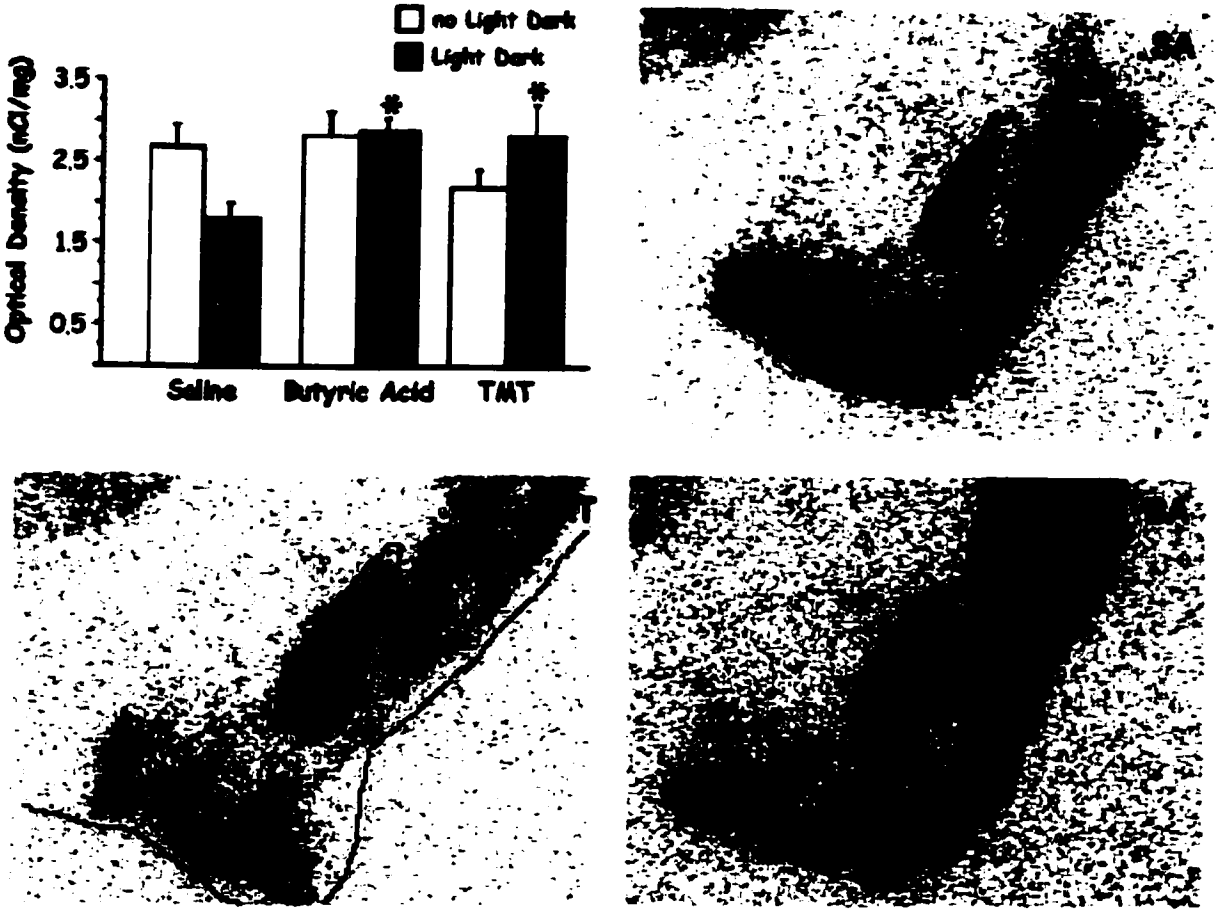
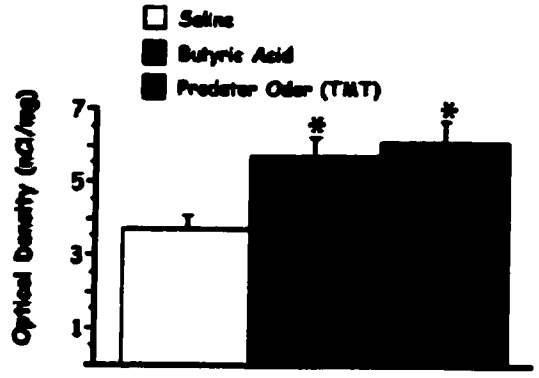
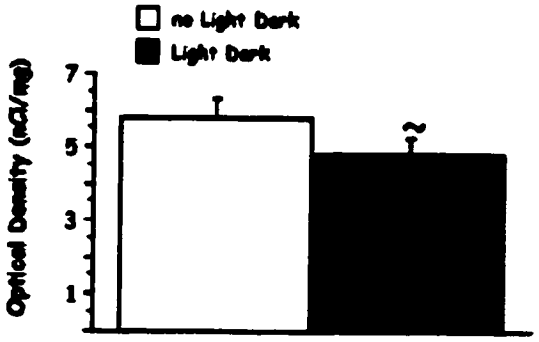
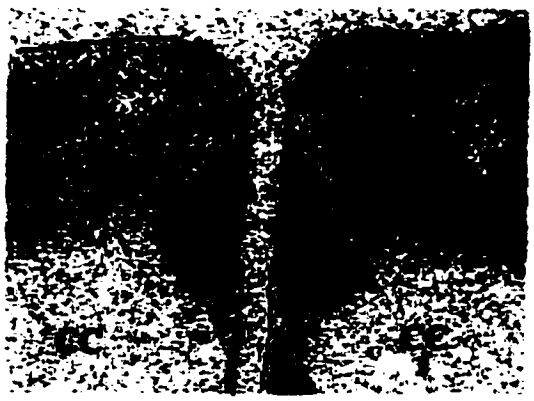


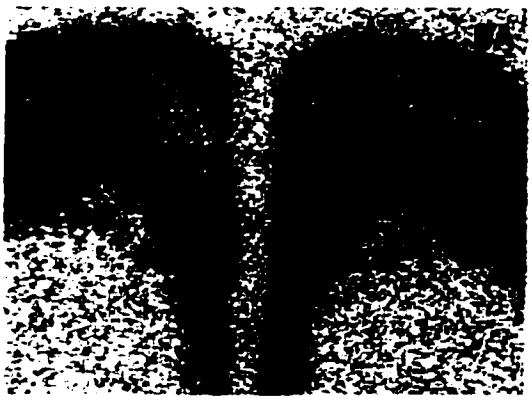
Figure 3.9: Cell mean (± S.E.M.) and representative photomicrographs for optical density (μCi/gr) of mPFC CCK mRNA among mice following a 10 minute saline (SA), butyric acid (BA) or fox odor (TMT) presentation (top panel) and among mice in light-dark and no light-dark testing schedules (bottom panel). Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treated mice while ~ depicts a statistically significant effect ($p < .05$) of light-dark box exposure. Note: CC= corpus callosum, Ci=cingulate cortex, PL=prelimbic cortex, IL=infralimbic cortex, black rectangle=representative sample area.

CCK mRNA


Saline
Butyric Acid
Predator Odor (TMT)
Optical Density (nCi/mg)
7
5
3
1
*
*


no Light Dark
Light Dark
Optical Density (nCi/mg)
7
5
3
1
≈





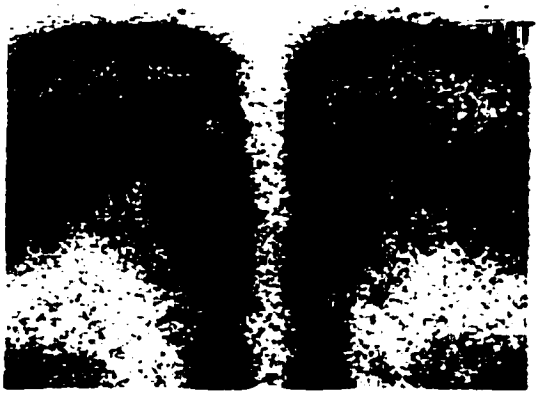
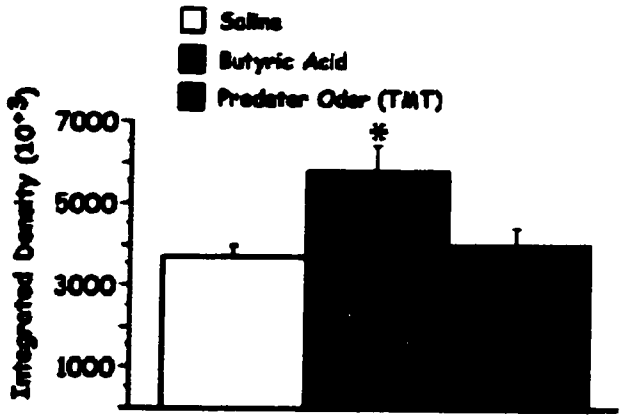
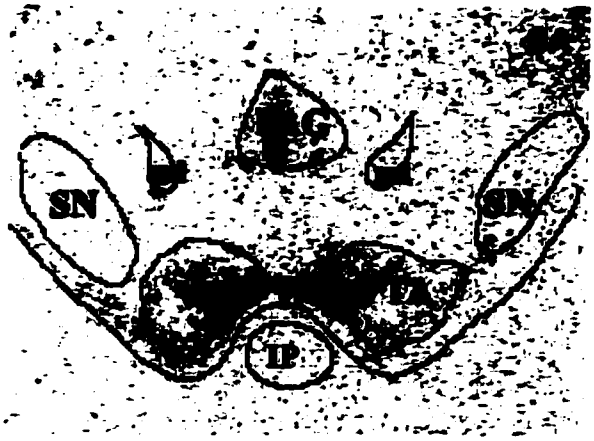
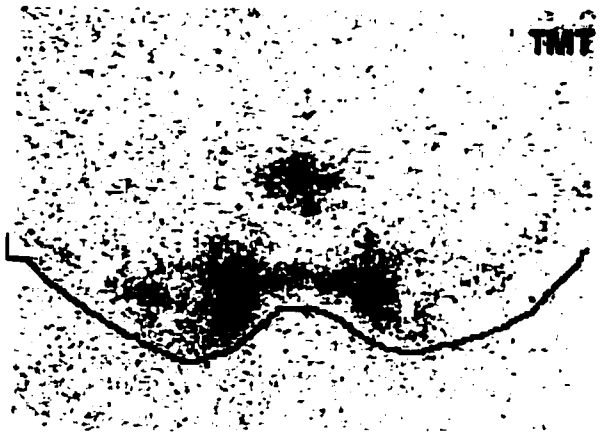


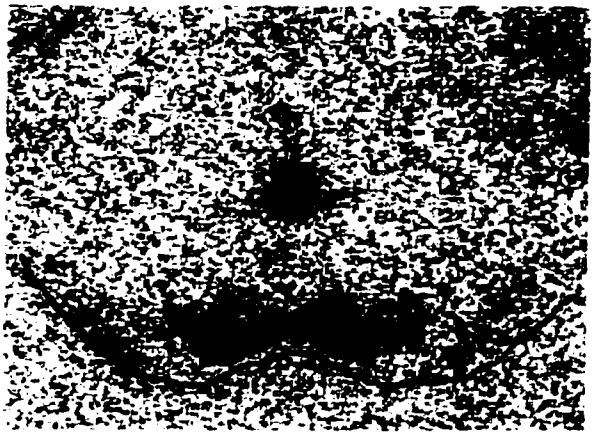
Figure 3.10: Cell mean (± S.E.M.) and representative photomicrographs for integrated density (μCi/gr * sq. pixel 10^3) of VTA CCK mRNA following a 10 minute saline (SA), butyric acid (BA) or fox odor (TMT) presentation among mice in light-dark and no light-dark testing schedules. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid presentation relative to saline or TMT treatment among mice irrespective of light-dark box condition. Note: VTA=ventral tegmental area, SN= substantia nigra, PAG= periaqueductal gray area, IF= interfascicular nucleus, ml= medial lemniscus.

Ventral Tegmental Area
CCK mRNA









DISCUSSION

The present study revealed that exposure of CD-1 mice to butyric acid or TMT in the home-cage induced risk assessment behaviour during odor presentation and subsequent anxiety in the light-dark box relative to mice exposed to saline. In particular, discrete mesolimbic CCK alterations were dependent on the propensity of either butyric acid or TMT to induce protracted indices of anxiety in the light-dark paradigm. While a 10 minute exposure of CD-1 mice to butyric acid was effective in enhancing CCK mRNA in the VTA irrespective of light-dark box activity, both butyric acid and TMT increased CCK mRNA in the mPFC. Mesolimbic CCK activity was associated with increased anxiety in the light dark box as revealed by increased CCK mRNA in the basolateral nucleus of the amygdala. It is likely that novel odors and predator odors, promote increased expression of CCK mRNA in the VTA and the mesocortex with concomitant decreases in exploratory tendencies while increased CCK gene expression in the basolateral regions of the amygdala are associated with gradient alterations in motivation and anxiety.

The behavioural pattern of defensive and non-agonistic behaviours following TMT, butyric acid or saline in the present investigation are consistent with previous investigations suggesting that a wide range of predator and non-predator odors elevate risk assessment and suppress non-defensive behaviours relative to neutral odor application during initial odor presentation (Blanchard et al., 1990; Garbe et al., 1993; Kemble & Bolwahnn, 1997). Mice exposed to butyric acid or the synthetic predator odor, TMT, contacted the odorant cloth less, displayed increased frequency of stretch attends, decreased rearing behaviour and enhanced defensive burying relative to mice exposed to saline. Yet, there were no differences in stretch attends or rearing patterns among mice exposed to TMT or butyric acid. These data

are consistent with previous investigations examining the behavioural profiles of mice in response to cat odor. In these studies, while mice display increased risk assessment behaviours to predator odor they exhibit similar reactions to almost every other novel odor encountered (Blanchard et al., 1990; Kemble, 1994; Kemble & Bolwahnn, 1997). The stretched attend posture, as well as defensive burying, can be observed in rats in response to an object that incites both fear and curiosity, including electric prods, novel and predator odors and are responsive to benzodiazepine administration (Blanchard et al., 1990; Molewijk et al., 1995). It should be noted that defensive burying of electric prods increases with increased shock intensity (Blanchard et al., 1990). In effect, the presentation of novel odors and predator odors may induce a fear gradient that underlies the emergence and intensity of defensive behaviours in CD-1 mice.

Indeed, in the present experiment, TMT was more effective than butyric acid in eliciting defensive behaviours to the respective odor presentation. For example, TMT depressed the frequency and duration of cloth contact and elevated the frequency and duration of freezing and defensive burying among CD-1 mice, more effectively than butyric acid, relative to saline treated mice. Wallace and Rosen (2000) report that TMT dose dependently increased freezing in rats. These data are in contrast to a recent report by Morrow et al. (2000) who employed the same amount of TMT (35 µl) purchased from the same distributor (i.e., PheroTech), in a similar manner (i.e. filter paper), albeit in a novel environment (i.e., open field), to rats. TMT did not induce alterations in freezing behaviour, grooming, vertical or horizontal activity of rats. These differences are hard to reconcile but may be dependent on species variations as well as the exposure environment. For example,

laboratory rats are less reactive to predator odors than mice (Blanchard et al., 1991) or more simply adopt different defensive strategies (Hendrie et al., 1996). Moreover, although Morrow et al. (2000) applied TMT in an open field, rats were habituated to environmental novelty and measures were taken to decrease the anxiogenic influence of the environment (i.e., illuminated with red light). Indeed, illumination with translucent red light has been shown to reduce anxiety in the light-dark box (Costall et al., 1989). Finally, it should be considered that encountering predator-associated cues in the 'safe' home environment represents a more relevant threat (Hendrie et al., 1996; Wallace & Rosen, 2000) than experience of predator odors in a novel environment where the likelihood of immediate and future predator avoidance/escape is increased.

Exposure of CD-1 mice to either butyric acid or TMT induced anxiety in the light-dark paradigm. Mice exposed to either butyric acid or TMT took longer to reenter the light section of the apparatus relevant to mice exposed to saline. Mice exposed to either butyric acid or TMT also spent less time in the light section of the apparatus relevant to mice exposed to saline. These data depicting the effects of butyric acid and TMT in the light-dark paradigm are consistent with the previous observation that time in light, is more sensitive to the anxiogenic influence of stressor imposition relative to transition scores (Kilfoil et al., 1989; MacNeil et al., 1997). Moreover, the mere presentation of novel odors (i.e., pungent versus non pungent) is sufficient to induce anxiety in the light-dark paradigm. In previous investigations, Hogg and File (1994) classified rats as responders or non-responders to cat odor based upon the time sheltering during cat odor exposure. Both cat odor responders and non-responders displayed anxiety in the elevated plus maze. In comparison, exposure of mice to TMT produced a profile of anxiogenic effects that was paralleled in both transition scores

as well as cumulative time in light. Mice exposed to TMT displayed decreased light and dark compartment transitions relevant to mice exposed to butyric acid or saline. The decrease in transition frequency is an index of reduced exploration (Crawley & Goodwin, 1980). Exposure of rats and mice to predators (Adamec, 2001) and predator odors (Berton et al., 1998) decrease exploration in novel environments. Mice exposed to TMT spent less time in the lit portion of the apparatus relative to mice exposed to either saline or butyric acid. The exaggerated behavioural effect of TMT on time spent in the light was delayed and did not emerge until the second concurrent 5 minute test session. The emergence of exaggerated levels of anxiety in mice exposed to TMT suggests a gradual divergence of novel and species-relevant fear-evoking stimuli in inciting behavioural change.

In the present investigation, CCK mRNA was increased from the mesocortex following exposure of mice to butyric acid (57 %) or predator odor (73 %) relative to saline exposure. Unexpectedly, exposure of CD-1 mice to the light-dark box reduced CCK mRNA values relative to mice not exposed to the apparatus irrespective of odor treatment. In other investigations, rat cerebral cortex preproCCK mRNA levels were increased after a single injection of diazepam (Rattray et al., 1993). In contrast, CCK mRNA was elevated in the VTA of mice exposed to butyric acid but not following TMT exposure relative to saline treated mice suggesting that the VTA is more sensitive to stressor applications compared to the prefrontal cortex. Indeed, butyric acid failed to induce freezing behaviour relative to saline or TMT treated mice yet was associated with increased CCK mRNA in the VTA. Moreover, the increase in VTA CCK mRNA among mice exposed to butyric acid was not associated with anxiety in the light-dark box. In previous investigations, a 10 minute exposure of rats to the visual and olfactory cues associated with footshocked conspecifics enhanced VTA DA

turnover, while a 30 minute exposure was required to effect mPFC DA turnover (Kaneyuki et al., 1991). It should be underscored that while exposure of mice to butyric acid failed to incite freezing in response to odor presentation defensive burying and stretch attends were increased relative to saline treated mice. It has been suggested that mesencephalic CCK plays an important role in maintaining the responsiveness of DA neurons to afferent input and behaviorally relevant stimuli (Hamilton & Freeman, 1995). Moreover, exposure to novel stimuli evoked investigatory activity and increased mesoaccumbens dopamine in rats. The phasic elevations in nucleus accumbens dopamine evoked by exposure to unconditioned novel stimuli were dependent on glutamate transmission in the VTA (Hooks & Kalivas, 1995; Legault & Wise, 2001; Vos et al., 1999).

Consistent with the demonstration that exposure of CD-1 mice to butyric acid or TMT induces anxiety in the light-dark paradigm as indicated by reduced cumulative time in light and longer light reenter latency, mice exposed to these odors displayed increased CCK mRNA in the basolateral nucleus (BLA) of the amygdala. It should be underscored that the increase in BLA CCK mRNA was evident only among those mice that were tested in the light-dark paradigm following respective odor treatment. The presentation of odors alone was ineffective in altering CCK mRNA within the BLA. In effect, the expression of anxiety in the light-dark box, and not the odor experience per se, was instrumental in inciting CCK alterations within this site. Taken together, these data suggest that mere odor presentation and not anxiety, per se, is associated with enhanced CCK mRNA in the mPFC and VTA while anxiety in the light dark box, irrespective of odor imposition, is associated with enhanced BLA CCK mRNA.

The basolateral amygdaloid nuclei participates in fear conditioning in human (Furmark et al., 1997) and infrahuman (Davis, 1992; Gewirtz et al., 1997; Graeff et al., 1993) subjects and may well assign motivational and emotional significance to stimuli previously paired with stressors (Davis & Shi, 1999; Morgan & LeDoux, 1995). In rats, lidocaine associated inactivation or electrolytic lesions of the basolateral nucleus of the amygdala prevented consolidation of the emotional learning and conditioning of acoustic startle (Gewirtz et al., 1998) and anxiety associated with repeated plus maze exposures (File et al., 1998). In humans (Davidson & Irwin, 1999) and animals (Granon et al., 2000; Jinks & McGregor, 1997; Morgan & LeDoux, 1995), the amygdala and mesocortex appear to participate in the acquisition and retention of emotionally laden stimuli. The basolateral nucleus of the amygdala plays a significant role in affective behaviour that is thought to be regulated by dopamine (DA) afferents from the mPFC. It has been demonstrated that the mPFC attenuates BLA neuronal activity via recruitment of BLA interneurons that suppress cortical inputs (Rosenkranz & Grace, 2001). Acute exposure of rats to TMT in an open field prompted increased DA metabolism, as measured by DOPAC/DA ratio, in the mPFC and basolateral amygdala and increased serum corticosterone. No significant effect of TMT was noted in the core and shell of the nucleus accumbens or the striatum. In contrast, butyric acid did not influence DA metabolism from any of the regions examined (Morrow et al., 2000). It should be noted that gentle handling also increased in vivo DA release from the basolateral aspects of the amygdala. Interestingly, such amygdaloid DA release was only evident during mild stressor application (Lanca et al., 1998).

In conclusion, the data of the present investigation reveal that CCK activation within the mesolimbic system, the basolateral nucleus of the amygdala in particular, following

stressor imposition contributes to an animals emotional state and alters behaviour accordingly. Indeed, one of the mechanisms associated with the expression of anxiety incorporates an amygdaloid DA/CCK neurochemical mosaic which may encode the saliency of the environmental experience, consistent with an amygdaloid dependent modulation of emotional memory (Davis, 1994). It should be considered that the presentation of butyric acid or TMT was associated with discrete mesocortical CCK alterations in CD-1 mice owing to the perceived severity of stressor-like properties associated with the respective odor. Indeed, mild stressor exposure among infrahuman subjects, ordinarily associated with alterations of DA and CCK sub-regions of the mesencephalon and mesocortex, induce gradient levels of anxiety while increased CCK availability in the basolateral amygdala is associated with increased vigilance and protracted indices of fear. In effect, sustained elevation of central CCK has been linked to the protracted impact of stressors on motivation and anxiety in clinical samples.

EXPERIMENTS 4, 5 AND 6

There is considerable evidence to suggest that exposure to aversive life experiences contribute to the constellation of behavioural disturbances among animals (Anisman et al., 1991). Some investigators argue that uncontrollable stressors incite behavioural change by promoting psychopathology, including anxiety and motivational change (McBlane & Handley, 1994). It is well documented that endogenous opioids contribute to the expression of affect and motivation in human (Castilla-Cortazar et al., 1998; Cohen et al., 1984; Zis et al., 1985) and infrahuman subjects (Hernandez et al., 1997). Indeed, major affective disorder has been associated with decreased cerebrospinal (CSF) endorphin concentrations (Berger & Nemeroff, 1987; Djurovic et al., 1999). Endogenous opioids have been implicated in the

mechanism of action of antidepressant therapies (DeFelipe et al., 1985; 1989). Chronic imipramine treatment promoted the expression of the μ- receptor in the hippocampus and frontal cortex of the rat (de Gandarias et al., 1998) and inhibited the enkephalin-degrading aminopeptidase in a concentration-dependent manner in rat brain (De Gandarias et al., 1997; Gallego et al., 1998). Likewise, among infrahuman subjects enkephalinase inhibitors possess antidepressant properties (Tejedor-Real et al., 1998), the therapeutic efficacy of various antidepressant agents are attenuated by the μ/δ receptor antagonist, naloxone (DeFelipe et al., 1985, 1989) and chronic antidepressant administration increases central enkephalin availability in the mesencephalon (Dziedzicka-Wasylewska & Papp, 1996). In contrast, mice deficient in the enkephalin precursor gene (i.e., prepro-enkephalin) display increased fear and anxiety in an open field and the elevated plus maze as well as increased aggression to conspecifics (Kieffer, 1999; Konig et al., 1996). In a similar vein, the opioid receptor antagonist, naltrexone, has been reported to increase aggression and blood pressure in an individual with post-traumatic stress disorder (Ibarra et al., 1994) and induce panic attacks in individuals with panic disorder (Maremmani et al., 1998).

Endogenous opioids have been implicated in diverse behavioural manifestations associated with stressor exposure, modulate defense responses (Blanchard et al., 1991) and incite coping behaviour among human and infrahuman subjects (Goodwin & Barr, 1997; Jamner & Leigh, 1999). Central administration of enkephalin agonists, administered either prior to or following neurogenic stressor encounter attenuated stressor-associated deficits in locomotor activity (Hebb et al., 1997) and brain stimulation (Zacharko et al., 1998) in mice. Moreover, naloxone attenuated the anxiolytic properties of diazepam and chlordiazepoxide in

mice in the light-dark box and elevated plus maze (Agmo et al., 1995; Belzung & Agmo, 1997; Tsuda et al., 1996). Among non-human primates, naloxone increased and morphine decreased vocalizations among infants separated from their mother (Kalin et al., 1988). These findings were replicated in rats exposed to a predator. In particular, morphine μ-receptor activation decreased (Shepherd et al., 1992) while naloxone increased (Blanchard et al., 1991) ultrasonic emissions in rats exposed to a cat. In particular, μ and δ neural activation may diminish anxiety and motivational loss accompanying stressor exposure, detract from the saliency of the stressful event and promote coping. Such processes may alter long-term responsivity of organisms to future stressor encounters.

Exposure of rats to predators and predator odor incites anxiety-like behaviour in the elevated plus maze (McGregor & Dielenberg, 1999), increased acoustic startle (Adamec et al., 1997; Plata–Salaman et al., 2000), increased freezing in novel environments (Hotsenspiller & Williams, 1997), and decreased sucrose consumption (Calvo-Torrent et al., 1999). Rats which are exposed to predator cues display enhanced risk assessment activity, including defensive burying, stretch attend and freezing behaviours (see Blanchard et al., 1998 for review; Dielenberg et al., 2001; Kemble & Bolwahnn, 1997) which are attenuated by diazepam and imipramine pretreatment (Blanchard et al., 1993; Grewal et al., 1997; Molewijk et al., 1995).

Predator odor exposure is associated with enhanced Fos production in the olfactory bulb, mesocortex, nucleus accumbens and central and medial amygdaloid nucleus (Funk & Amir, 2000; Dielenberg et al., 2001). Proto-oncogenes or immediate early genes (IEGs) such as c-fos, homologous genes of c-fos: fos related antigen (FRAs) (fos B, fra1 and fra2), c-jun, zinc finger family genes and NGFI-B are expressed immediately (e.g., 5-10 minutes) in

response to extracellular stimuli and play a fundamental role in signal transduction and transcriptional regulation of neuronal cells. Fos forms heterodimers with Jun proteins (i.e., Fos-Jun complex) known as AP-1 and binds to the AP-1 binding sites of target genes, including prepro-enkephalin among others, regulating transcription of enkephalin precursor peptides. The expression of FRAs can be induced by stimuli that up-regulate c-Fos expression. However, while Fos expression is transient, reaching an asymptote in 90 minutes, FRA expression follows a more protracted time-course, peaking 6-8 hours following acute stimulation and accumulating in the brain for days following repeated stimulation (Herrera & Robertson, 1996; Hope et al., 1994).

Exposure of animals to species specific predatory cues may provide a relevant simulation of clinical psychopathology. Moreover, equating the intensity of the predator and control odors strengthens behavioural interpretation. The application of odor substances extracted from the scent gland of the fox or the weasel, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) or 2-propythietane (PT), respectively, favors quantitative evaluation of experience intensity while permitting quantitative evaluation of non-predatory odor intensity (Crump & Moors, 1985; Lu et al., 1993; Vernet-Maury, 1980; Wallace & Rosen, 2001; Zibrowski et al., 1998). TMT and PT induce fear in rats as revealed by suppressed open-field activity and elevated corticosterone levels. TMT and PT also increase opioid dependent analgesia and freezing in rats relative to the pungent control odor, butyric acid (Hotsenpiller & Williams, 1997; Kavaliers et al., 1997; Kavaliers & Choleris, 1997) similar to rats exposed to cat (Lester & Fanselow, 1985; Lichtman & Fanselow, 1990) and weasel odor (Kavaliers, 1988). Finally, rats exposed to TMT display more freezing in an open field in the absence of habituation regardless of developmental stage (Vernet-Maury et al., 1992). The purpose of the present

investigations was to assess whether the mere presentation of predator odor is sufficient to incite central opioid variations in mesolimbic sites as well as behaviours associated with mild anxiety states and anhedonia. Indeed, it has been demonstrated that increased amygdaloid expression of prepro-enkephalin, accomplished by administration of a replication-defective herpes virus carrying human prepro-enkephalin cDNA delivered to rat amygdala enhanced the anxiolytic properties of diazepam in the elevated plus maze (Kang et al., 1999; 2000). Taken together, increased neuronal activation and prepro-enkephalin availability in mesolimbic sites is associated with mood elevation and coping ability which may detract from the aversiveness of the stressor experience.

Experiment 4 assessed the immediate defensive behavioural repertoire previously described in Experiment 3 among CD-1 mice acutely exposed to TMT as well as the immediate (e.g., 5 minutes) and long-term (e.g., 1 week) behavioural consequences of such substance exposure in the light-dark box. In situ hybridization and immunohistochemical analyses of prepro-enkephalin mRNA and/or FRA were also undertaken to compare the neurochemical signature associated with predatory exposure and the overt behavioural indices of fear and anxiety. Experiment 5 assessed the acoustic startle response in juvenile and mature CD-1 mice exposed to acute TMT both immediately (e.g., 5 minutes) and at a longer post-odor interval (e.g., 1 week). Experiment 6 evaluated the putative anhedonic consequences of TMT presentation on reward threshold alterations among CD-1 mice responding for brain stimulation from the dorsal aspects of the mesencephalon soon after psychogenic stressor encounter and following relatively protracted intervals. It has been previously been demonstrated in this laboratory that brain stimulation from ventral aspects of the VTA is stressor resistant (Hebb et al., 1997; Zacharko et al., 1998). While it has been well

documented that neurogenic stressors induce anhedonia in mice as revealed by attenuated responding for previously rewarding brain stimulation from mesolimbic areas (Zacharko & Anisman, 1991; Zacharko et al., 1998: Zacharko et al., 1990), the influence of psychogenic stressors on motivation remains to be determined.

MATERIALS AND METHODS

Subjects

Naive, male, 3 month old, CD-1, mice (N=241: Experiment 4, n=164; Experiment 5, n= 59; Experiment 6, n=18) were presented with 2,5-Dihydro-2, 4,5-trimethylthiazoline (TMT), butyric acid (Sigma) or saline as previously described in Experiment 3.

Light-Dark Apparatus

The light-dark apparatus was previously described in Experiment 1 and the identical experimental procedure in Experiment 3. Following habituation procedures, mice were randomly assigned to predator (n=57), butyric acid (n=53), or saline (n=54) treatment conditions. Twenty-four mice were then transported immediately (5 minutes) to the second experimental room where activity in the light-dark box was recorded (Immediate Group, n=8 for each of the 3 odor groups). All animals (N=164) were then given a new, clean home-cage and returned to the main animal housing area. Independent groups of subjects (N=18 per group) were used to assess light-dark performance 24h (n=6 per 3 odor conditions), 48h (n=6 per 3 odor conditions), and 168 h (n=6 per 3 odor conditions), following the initial odor exposure. In order to control for a possible interaction between odor presentation and light-dark box exposure on central neurochemical correlates, independent groups of mice (n=76) were exposed to either Predator (n=26), Butyric Acid (n=25), or Saline (n=25) and sacrificed Immediately (5min), 24 hours, 48 hours, or 168 hours. To assess the influence of relatively

abbreviated exposure on behaviour and immunohistochemistry, eight mice were exposed to either 5 minutes of predator exposure or 5 minutes of saline and perfused immediately. Five animals from each group were perfused intracardially with 4% paraformaldehyde (3.8% Sodium Borate) and brains removed for immunohistochemistry of FRA and in situ hybridization of prepro-enkephalin mRNA.

Immunohistochemistry/In Situ Hybridization

A combination of immunohistochemistry for FRA and in situ hybridization histochemistry for prepro-enkephalin (ENK) mRNA was performed in order to allow simultaneous visualization of FRA and ENK mRNA/ FRA in the same tissue section. Every fourth section was processed using the avidin-biotin bridge method with hydrogen peroxide as a substrate. The immunohistochemistry reaction was performed in RNAse-free conditions using rabbit antihuman/rat Fos-related antigen (FRA, K-25, Santa Cruz) that recognized FRA-1, FRA-2, Fos and Fos B at a concentration of 1:5000. Following the immunohistochemistry procedures, the sections were used for in situ hybridization with a 35S-labeled enkephalin cRNA probe for mRNA. The enkephalin cRNA probe (935 bp) was generated from a rat cDNA contained in pSP64 (antisense) and pSP65(sense) plasmids (Dr. S. Sabol, NIMH, Bethesda, MD).

In brief, the immunohistochemistry reaction was performed first in RNAse-free conditions followed by in situ hybridization. Briefly, sections were washed in sterile DepC-treated 0.05M potassium-buffered saline (KPBS) and incubated at 4°C with the FRA antibody mixed in sterile KPBS, 0.4% Triton X-100, 1% heparin sodium salt USP, and 1% bovine serum albumin. Approximately 18 hours after incubation with the FRA antibody (Santa Cruz, K-25) brain sections were rinsed in sterile KPBS and incubated with a mixture of

KPBS-heparine and biotinylated secondary antibody for 120 minutes. The sections were then rinsed with KPBS and incubated at room temperature for 60 minutes with an avidin-biotin-peroxidase complex (Vectastain Elite). After several rinses in sterile KPBS, the brain sections were then immersed in a mixture containing sterile KPBS, the chromagen 3,3'-diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/ml) and hydrogen peroxide (0.003%). Thereafter, hybridization histochemical localization of ENK mRNA was carried out using 35S-labeled cRNA probe (psp64 plasmid containing a 935-bp Sac I fragment of rat ENK cDNA, Dr. Sabol, NIMH). The PCR fragment of 604 bp was sub-cloned in vector pBluescript-SKII (±) using blunt-end method at the SMA1 site and digested with EcoR1 and SAC1 respectively, to produce antisense and sense probes.

Protocols for riboprobe synthesis, hybridization and autoradiographic localization of ENK mRNA signal were adapted from Simmons et al. (1989). Immunoreactive sections for FRA were mounted onto poly-L-lysine-coated slides and were desiccated under vacuum overnight, fixed in 4% paraformaldehyde for 30 minutes and digested by proteinase K (10 µg/ml in 100 mM tris HCl (pH 8.0), and 50 mM EDTA, at 37°C for 25 minutes). The brain sections were then rinsed in sterile DepC treated water followed by a solution of 100 mM triethanolamine (TEA, pH 8.0) acetylated in 0.25% acetic anhydride in 100 mM TEA and dehydrated through graded concentrations of alcohol (50, 70, 95, and 100%). After vacuum drying for a minimum of 2 h, 90 µl of hybridization mixture (107 cpm/ml) was spotted on each slide, sealed under a coverslip and incubated at 57°C overnight (~15-20 h) in a slide warmer. Coverslips were then removed and the slides were rinsed in 4x standard saline citrate (SSC) at room temperature. Sections were digested by RNAse A (20 µg/ml, 37°C, 30

minutes), rinsed in descending concentrations of SSC (2x, 1x, 0.5x SSC), washed in 0.1x SSC for 30 minutes at 60°C (1x SSC: 150 mM NaCl, 15 mM trisodium citrate buffer, pH 7.0) and dehydrated through graded concentrations of alcohol. After being dried for 2 h under vacuum, the sections were defatted in xylene and dipped in NTB2 nuclear emulsion (Kodak; diluted 1:1 with distilled water). Slides were exposed for approximately 7 to 10 days, developed in D19 developer (Kodak) for 3.5 minutes at 14-15°C and fixed in rapid fixer (Kodak) for 5 minutes. Thereafter, tissues were rinsed in running distilled water for 1 to 2 h and dehydrated through graded concentrations of alcohol, cleared in xylene and coverslipped with DPX.

Data Analysis

Odor Presentation and Light-Dark Paradigm

The home-cage behavioral analysis and behavioural scores pertaining to light dark box exposure were analyzed as previously described in Experiment 3.

Anxiety in the Light-Dark Box

According to the total time spent in the light chamber of the apparatus irrespective of odor treatment or test session, mice were termed "anxious" or "non-anxious". Anxious mice spent 300 seconds or less in the light chamber of the light dark box while non-anxious mice spent greater than 50% of the total apparatus time in the light chamber of the box.

Predator Odor Responders and Non-responders

According to their behavioural response in the home-cage to predator odor, mice were classified as "responders" or "non-responders" to odor treatment based on the duration of freezing behaviour. Responders displayed freezing in excess of 1 minute while mice that displayed decreased freezing durations (< 1 minute) in response to odor presentation were classified as non-responders. Analysis of variance of the total time in light was employed to

determine the effect of fox odor on anxiety in the light-dark box over test sessions in CD-1 mice. Fisher's least significant difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

In Situ Hybridization and Immunohistochemistry: ENK and FRA

Nuclei were identified using the Paxinos and Franklin mouse brain atlas (1997). For each animal, the amygdala sections corresponding to level 43-44, the medial prefrontal cortex corresponding to levels 14-19, the shell and core of the nucleus accumbens corresponding to levels 19-24 and the dorsal aspects of the VTA corresponding to levels 60-62 were examined under bright field (to reveal FRA perikarya), as well as darkfield (to distinguish ENK–expressing cell bodies) microscopy. An eyepiece was fitted with a 1 cm x 1 cm grid square to delineate the area and isolate individual cells to count. FRA and ENK cell bodies were counted. In addition, blind quantitative analysis of hybridization signal for ENK mRNA was performed on X-ray film (Kodak) as previously described in Experiment 1. The number of positively stained cells were counted in the amygdala, VTA, nucleus accumbens and mesocortex and subjected independently to ANOVA as a 3 (Fox, Butyric Acid, Saline) x 4 (Immediate, 24h, 48h and 168h) x 2 (light-dark box or no light-dark box) design in mice following a 10 minute odor presentation. Separate analyses were conducted for FRA and ENK in (a) mice tested in the light dark paradigm or merely exposed to the respective odor (b) mice exposed to abbreviated odor presentations (c) mice classified as responders or non-responders to predator odor and (d) mice termed anxious or non-anxious in the light dark box. Fisher's least significant difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

RESULTS

Home Cage Exposure

Analysis of variance of the frequency of (a) contacts with the odorant cloth, $F(2, 161)=131.41$, $p<.001$) (b) freezing $F(2, 161 =75.425$, $p<.001$), (c) defensive burying $F(2, 161 =44.924$, $p<.001$), (d) stretch attends $F(2, 161 =32.633$, $p>.001$) (e) grooming $F (2, 161 =7.347$, $p>.001$) and (f) rearing $F(2, 161 =51.422$, $p>.001$) among mice following odor presentation revealed a main effect of odor. (See Figure 4.1 and Figure 4.2).

Insert Figures 4.1 and 4.2 about here

Analysis of variance of the duration of (a) contact with the odorant cloth $F(2, 161 =148.98$,$p<.001$) (b) freezing $F(2, 161 =41.247$, $p<.001$) and defensive burying $F(2, 161 =36.970$, $p<.001$) revealed a main effect of odor (See Figure 4.3).

Insert Figure 4.3 about here

Light-Dark Paradigm

Analysis of variance of the time spent in the light during the first and second 5 minute intervals of the light dark box among mice following odor exposure, revealed a main effect of odor during the immediate test session only, $F(2, 21) =6.57$, $p<.01$ and $F(2, 21) =6.80$, $p<.01$, respectively (See Figure 4.4). ----------

Insert Figure 4.4 about here

Figure 4.1: Home cage behavioral frequency (± S.E.M.) exhibited among mice during a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treatment while ** depicts a statistically significant difference ($p < .05$) among butyric acid and TMT odor treatments for a particular home-cage behavior observed during odor presentation.

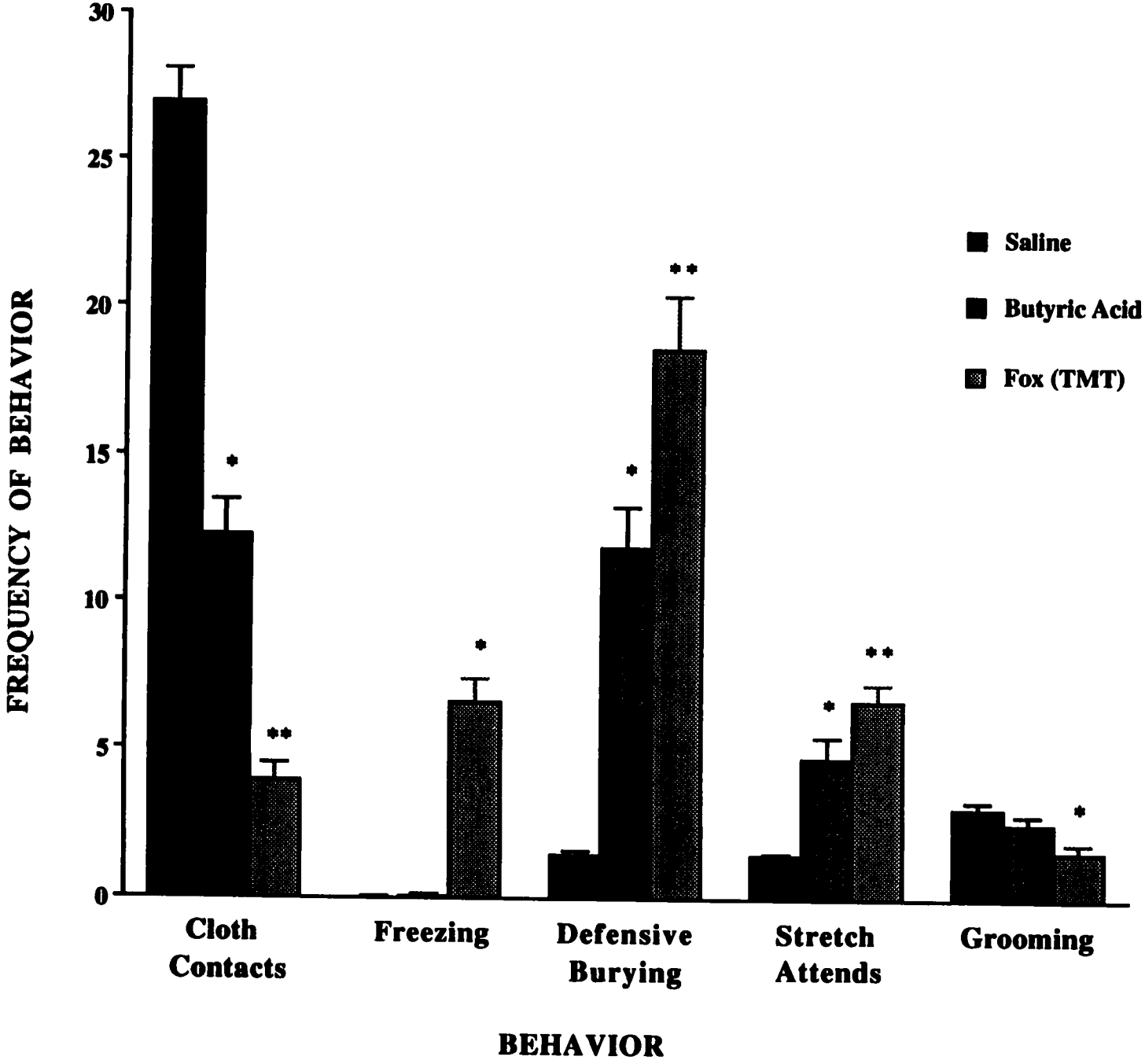

30
25
20
15
10
5
0
FREQUENCY OF BEHAVIOR
Saline
Butyric Acid
Fox (TMT)
*
**
Cloth Contacts
Freezing
Defensive Burying
Stretch Attends
Grooming
BEHAVIOR

Figure 4.2: Home cage rearing frequency (± S.E.M.) exhibited among mice during a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts statistically significant difference ($p < .05$) of butyric acid or TMT odor treatments relative to saline treated mice while ** depicts statistically significant difference ($p < .05$) between butyric acid and TMT odor treatments on rearing behavior observed during odor presentation.

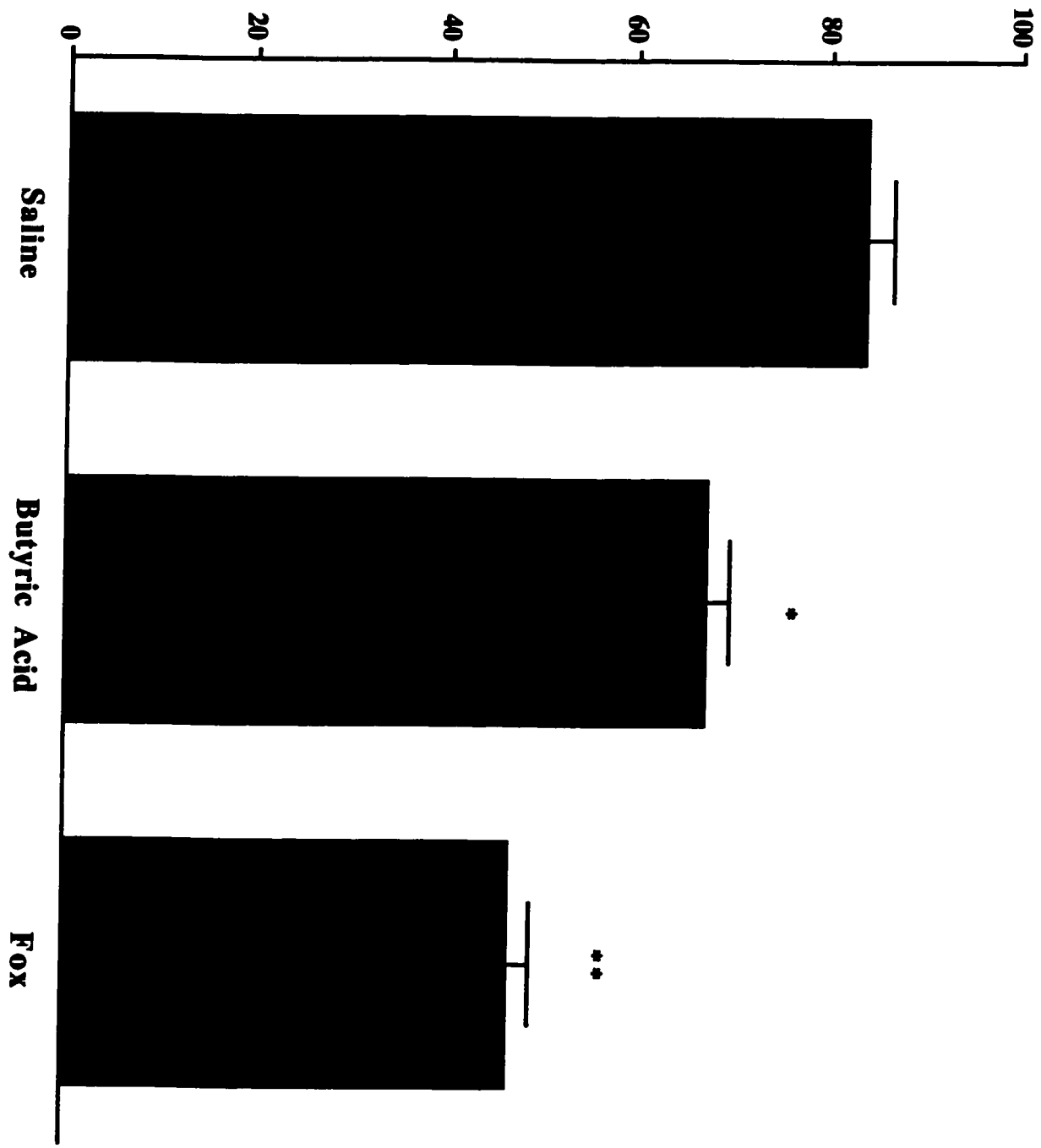

FREQUENCY OF REARING
0
20
40
60
80
100
Saline
Butyric Acid
Fox
*
**

Figure 4.3: Duration of home-cage defensive behaviors (± S.E.M.) exhibited among mice during a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treatment while ** depicts statistically a significant difference ($p < .05$) among butyric acid and TMT odor treatments for a particular home-cage behavior observed during odor presentation.

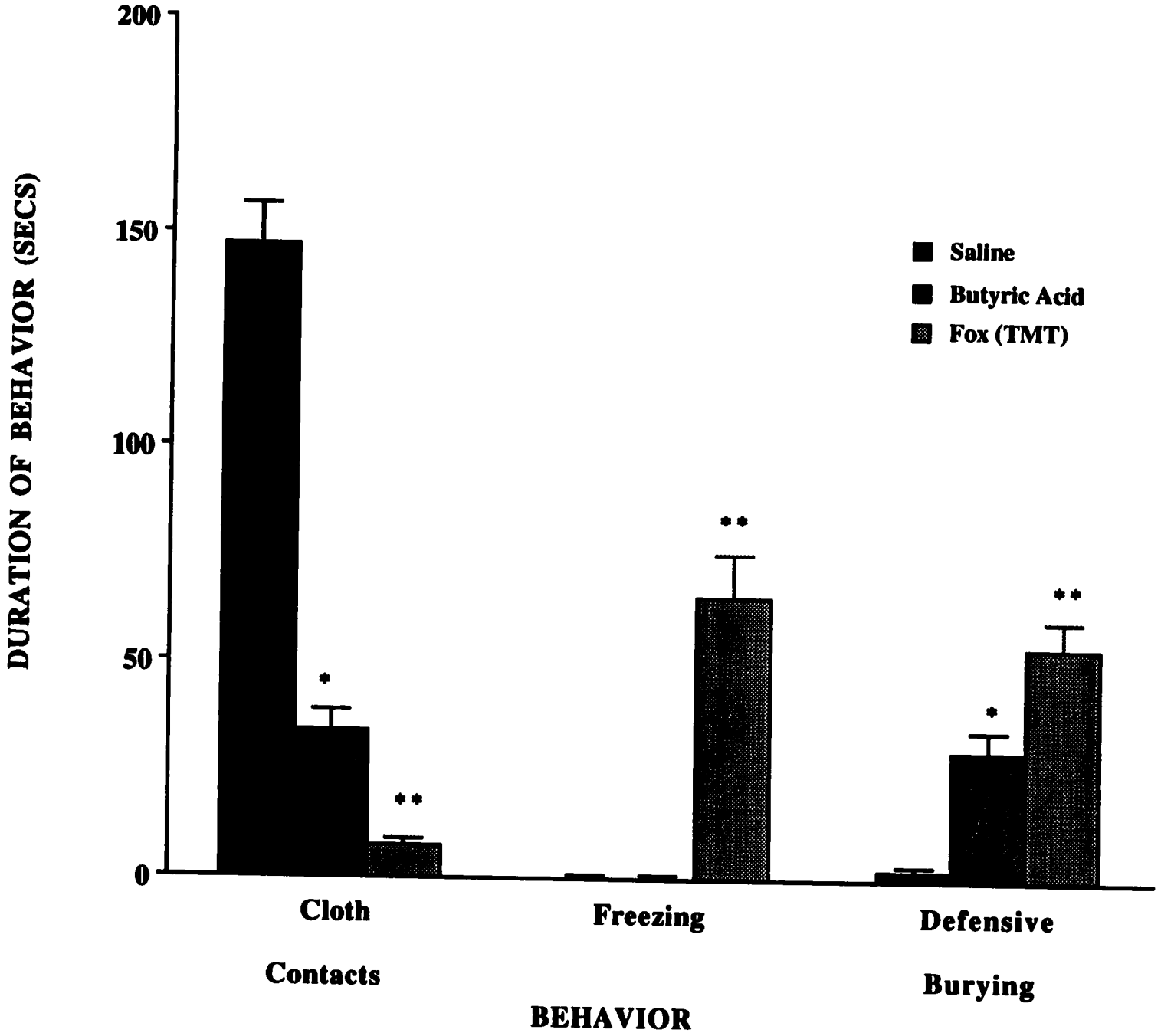
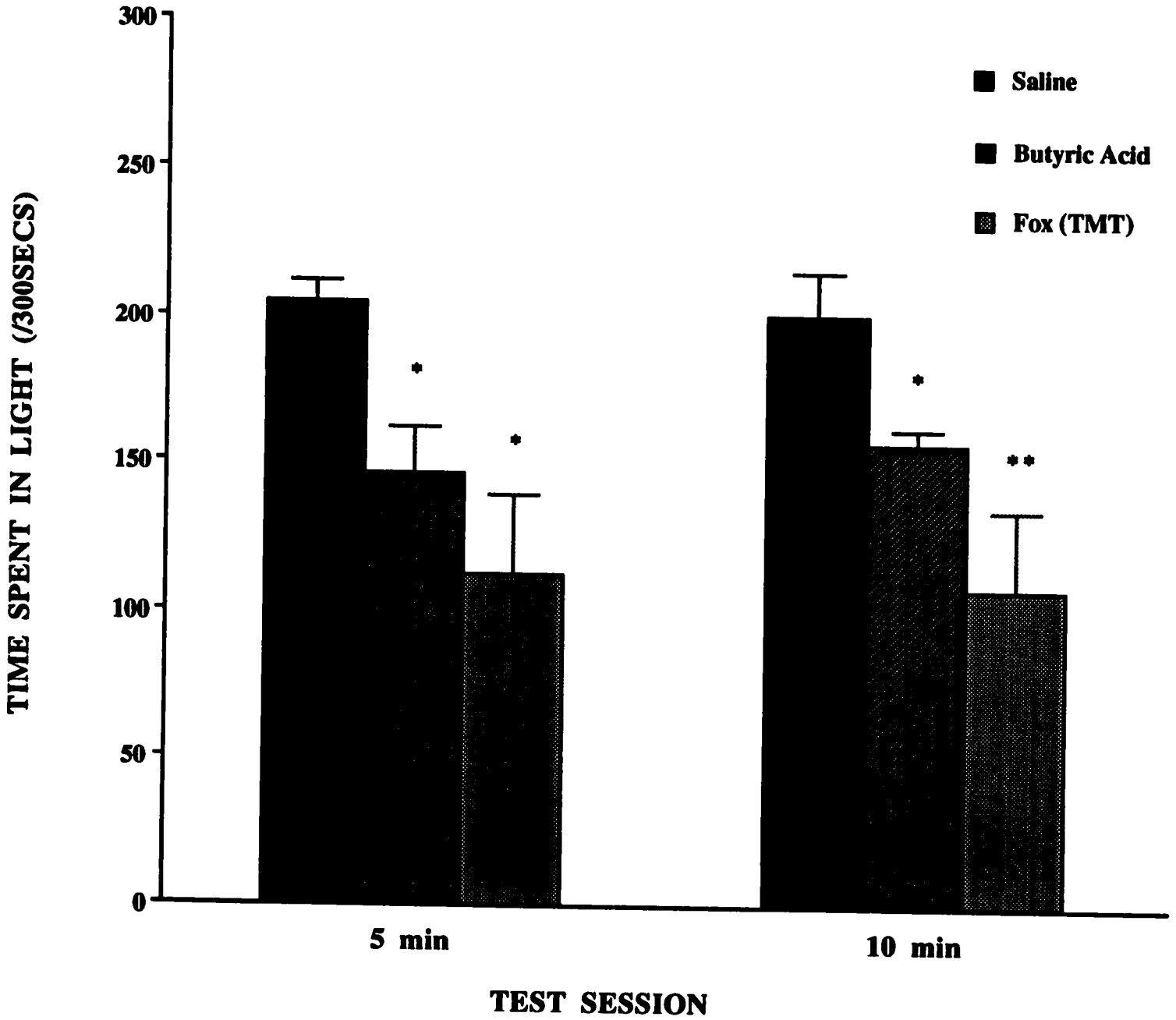

200
150
100
50
0
DURATION OF BEHAVIOR (SECS)
Saline
Butyric Acid
Fox (TMT)
*
**
**
*
**
Cloth
Contacts
Freezing
Defensive
Burying
BEHAVIOR

Figure 4.4: Mean (± S.E.M.) time spent in the light chamber of the light dark box during the first and second consecutive 5 minute intervals in mice immediately following a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid and TMT odor treatment relative to saline treatment while ** depicts a statistically significant difference ($p < .05$) among butyric acid and TMT odor treatments at the identical post-stressor interval. These data were presented in Experiment 3.


300
250
200
150
100
50
0
TIME SPENT IN LIGHT (/300SECS)
Saline
Butyric Acid
Fox (TMT)
*
*
*
**
5 min
10 min
TEST SESSION

Analysis of variance of light chamber reentry latency among mice following odor exposure revealed a significant effect of odor during the immediate test session only, $F(2, 21) = 3.87$, $p < .05$ (See Figure 4.5).

Insert Figure 4.5 about here

Analysis of variance of the cumulative time spent in the light chamber of the light dark box among mice following odor exposure revealed a main effect of odor during the immediate test session only $F(2, 21) = 7.63$, $p < .01$ (See Figure 4.6).

Insert Figure 4.6 about here

Analysis of variance of cumulative light and dark compartment transitions of CD-1 mice revealed a significant effect of odor immediately following odor exposure, $F(2, 21) = 3.66$, $p < .05$ (See Figure 4.7).

Insert Figure 4.7 about here

Analysis of variance of dark chamber latency and rears in the light chamber among CD-1 mice on the immediate day failed to reach statistical significance. Analysis of variance of dark chamber latency, light-dark chamber transitions, time in light chamber or light chamber reentry of CD-1 mice failed to reach statistical significance 24, 48 or 168 hours following odor presentation (data not shown).

Figure 4.5: Light chamber reentry latency (± S.E.M.) of mice following initial dark compartment escape immediately following a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treated mice.

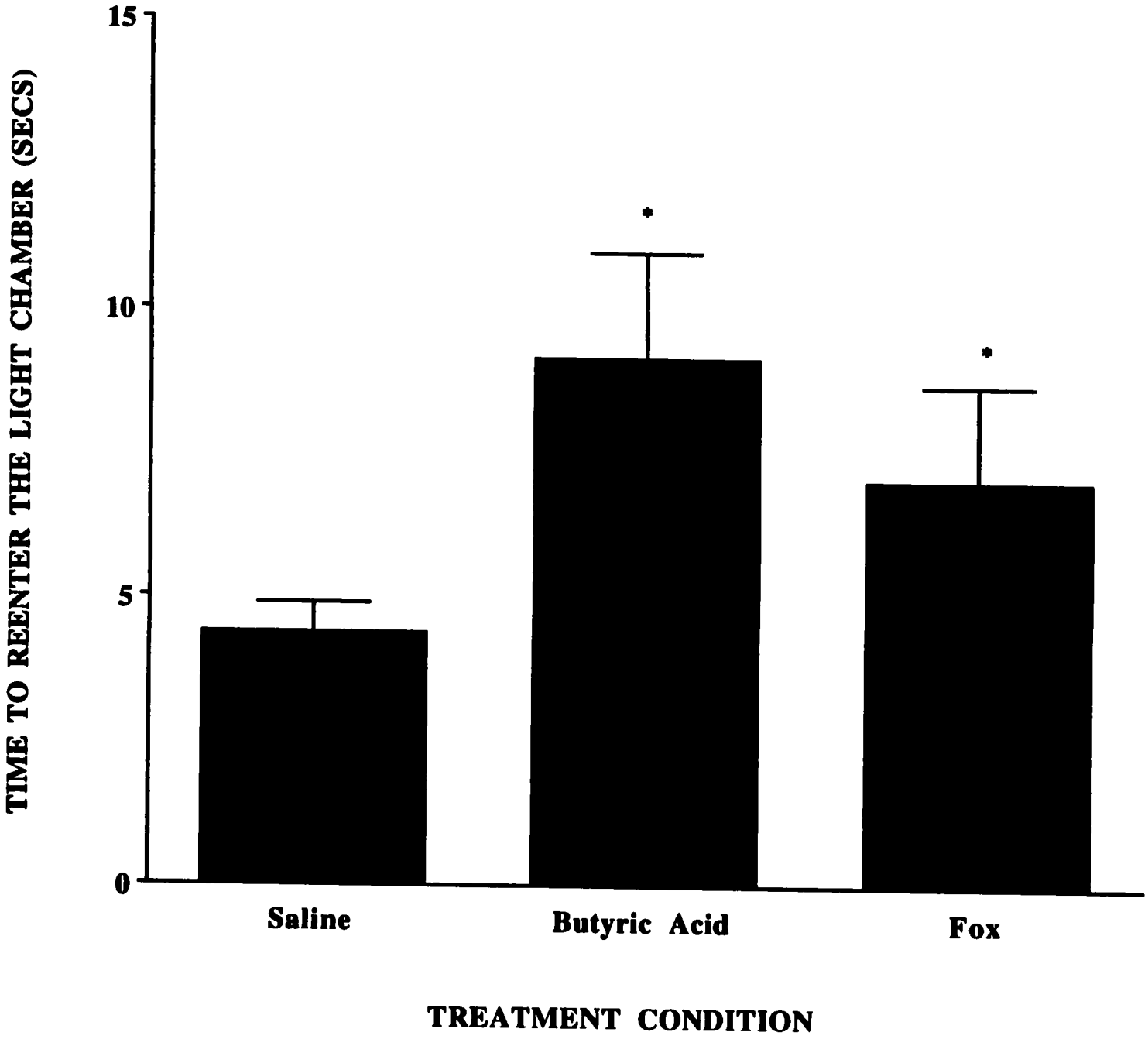

15
10
5
0
TIME TO REENTER THE LIGHT CHAMBER (SECS)
*
*
Saline
Butyric Acid
Fox
TREATMENT CONDITION

Figure 4.6: Mean (± S.E.M.) cumulative time spent in the light chamber of the light-dark box among mice following a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of butyric acid or TMT odor treatment relative to saline treated mice. These data were presented in Experiment 3.

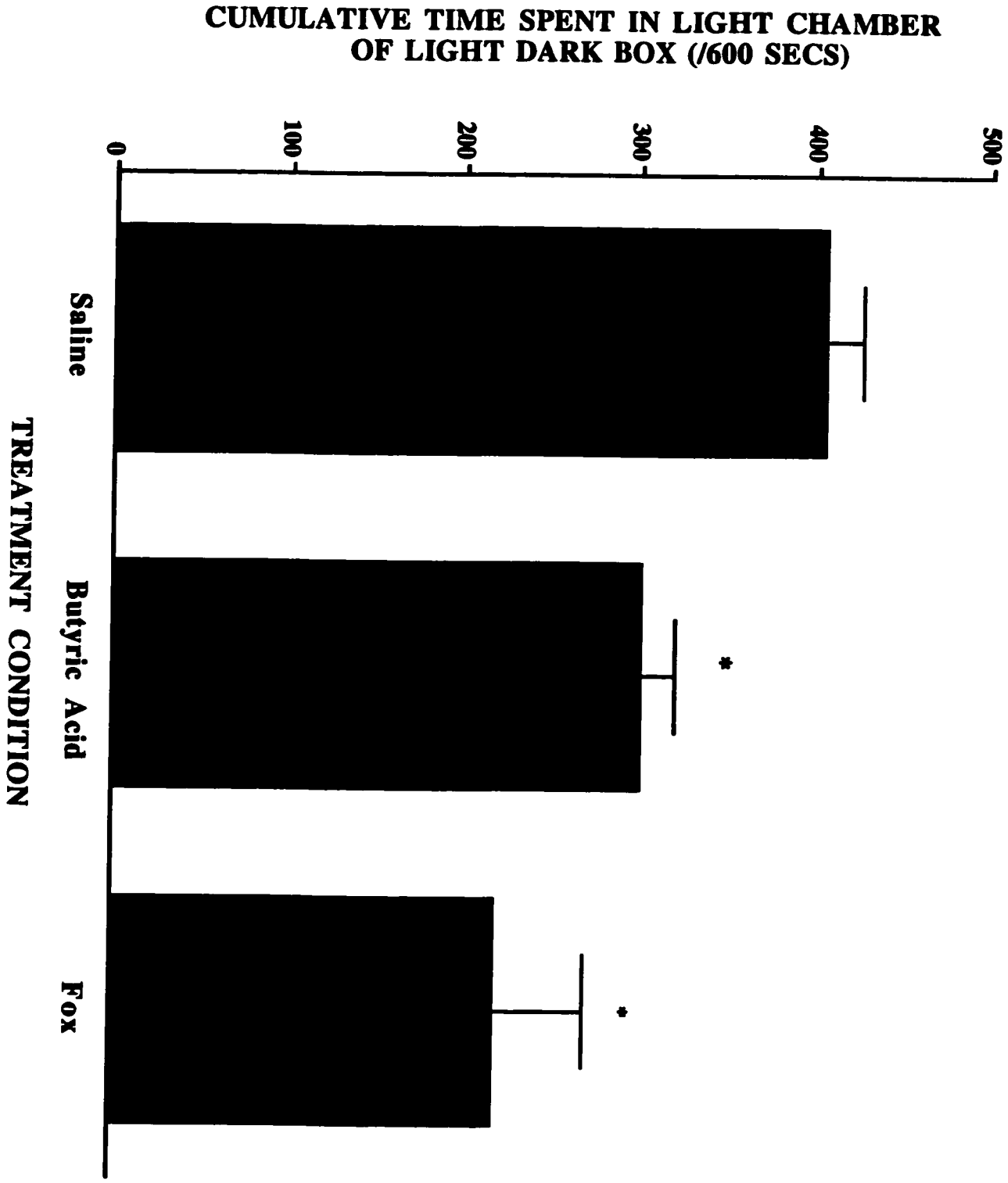
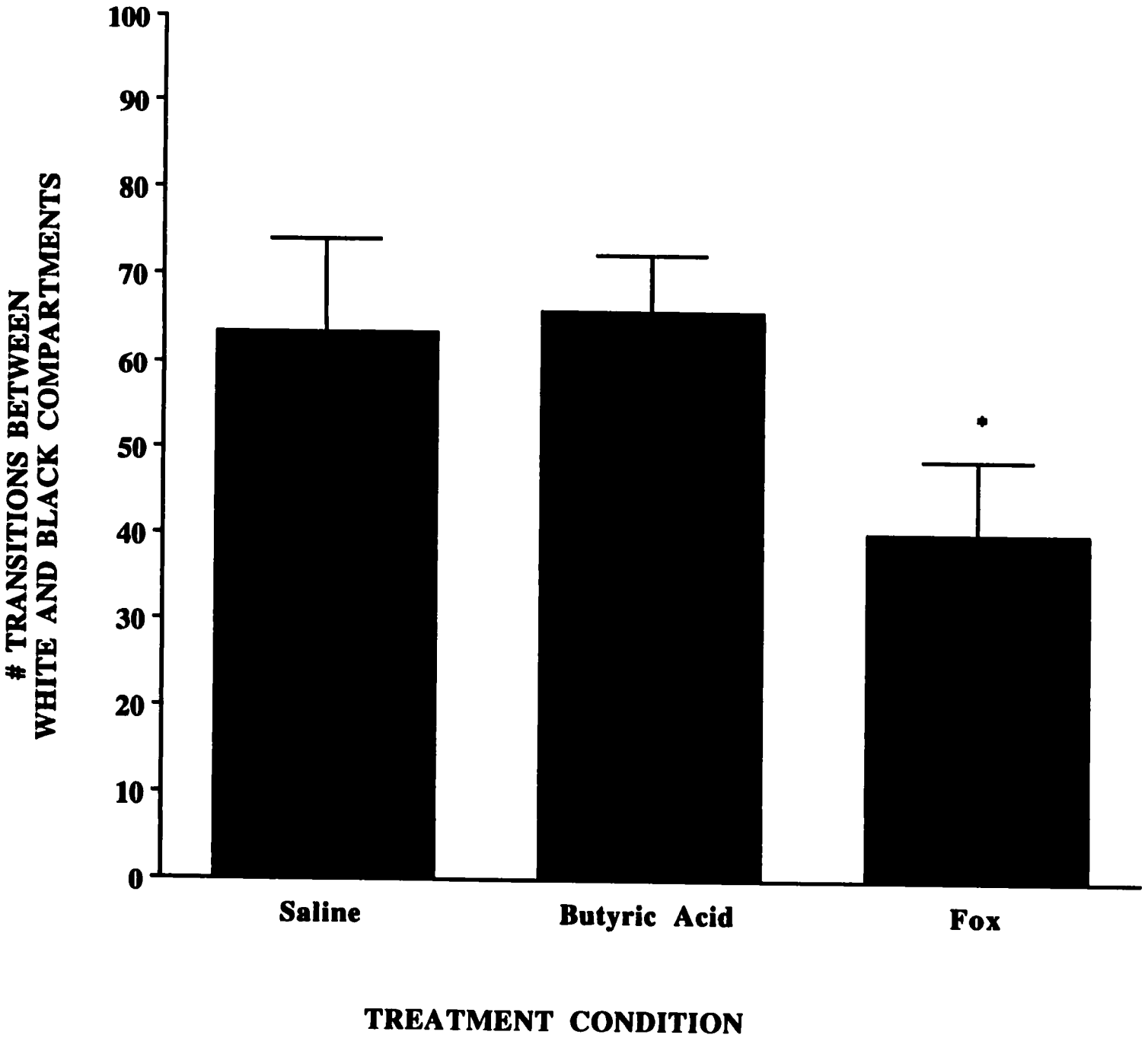

CUMULATIVE TIME SPENT IN LIGHT CHAMBER
OF LIGHT DARK BOX (/600 SECS)
0
100
200
300
400
500
Saline
Butyric Acid
Fox
*
*
TREATMENT CONDITION

Figure 4.7: Cumulative (± S.E.M.) light and dark compartment transitions of mice immediately following a 10 minute saline, butyric acid or fox odor (TMT) presentation. Note: * depicts a statistically significant difference ($p < .05$) of TMT odor treatment relative to saline treatment. These data were presented in Experiment 3.


100
90
80
70
60
50
40
30
20
10
0
TRANSITIONS BETWEEN
WHITE AND BLACK COMPARTMENTS
*
Saline
Butyric Acid
Fox
TREATMENT CONDITION

Analysis of variance of the total time spent in the light chamber of the apparatus in predator-treated mice classified as responders or non-responders over the four test sessions revealed a main effect of odor response, $F(1, 22) = 4.775$, $p<.05$ and an odor response x test session interaction $F(1, 22) = 4.775$, $p<.05$. Mice classified as odor responders based on increased duration of freezing behaviour during original predator odor presentation spent less time in the light chamber of the light-dark box than mice that displayed decreased freezing durations during odor presentation. This effect was most pronounced during the immediate test session (See Figure 4.8).

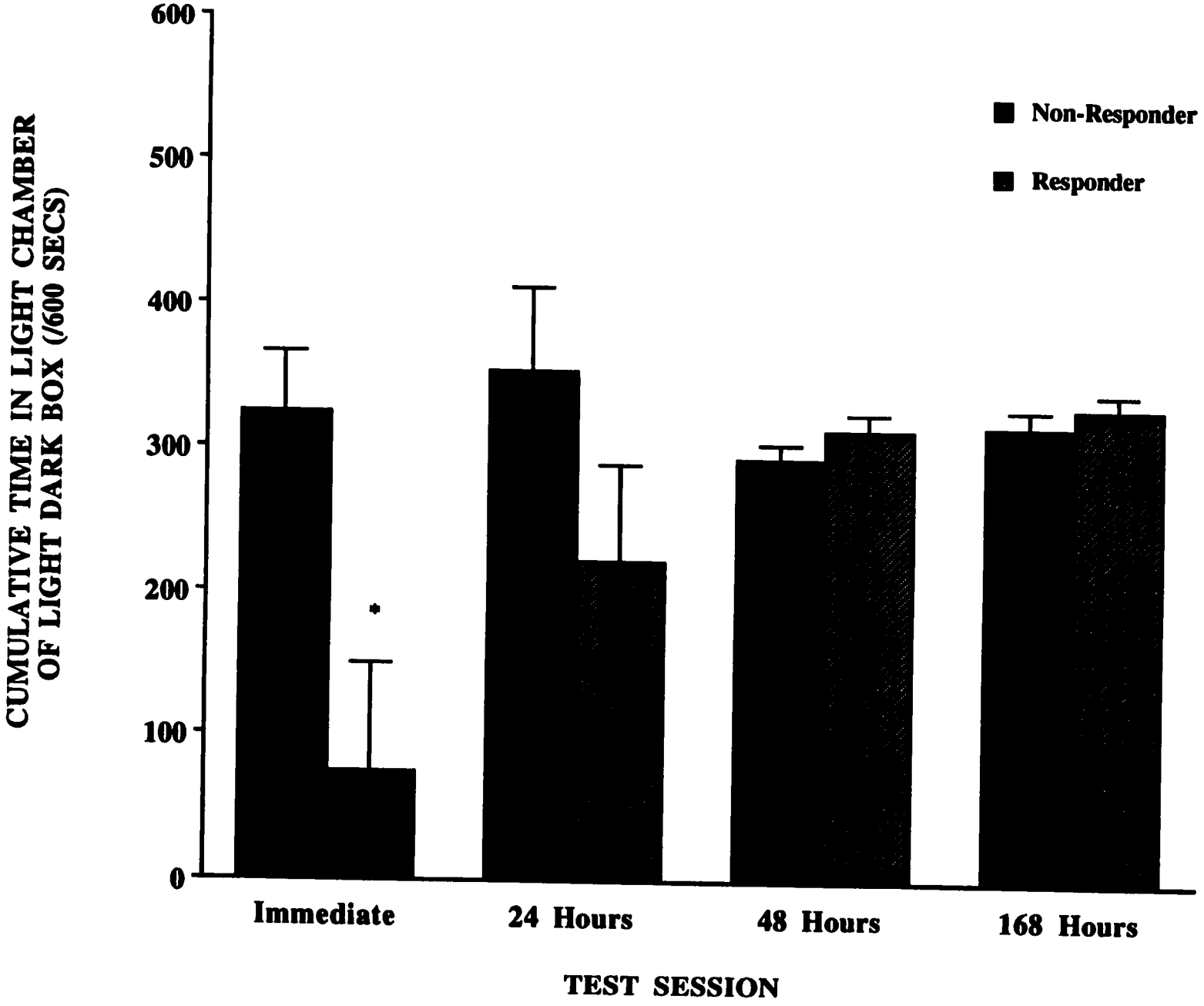
Insert Figure 4.8 about here

FRA Immunohistochemistry

Medial prefrontal cortex:

Analysis of variance of FRA cell counts in the prelimbic cortex among mice following odor exposure revealed a main effect of odor $F(2, 89) = 15.258$, $p<.001$, a main effect of test session $F(3, 89) = 28.022$, $p<.001$ and a main effect of light-dark box exposure $F(1, 89) = 4.318$, $p<.05$. Exposure of mice to TMT or the light dark box increased FRA relative to saline and butyric acid or mice in the No LD condition, respectively. These changes were most prominent on the immediate test session. Analysis of variance of FRA cell counts in the infralimbic cortex revealed a main effect of odor $F(2, 91) = 14.978$, $p<.001$ and a main effect of test session $F(3, 91) = 2.787$, $p<.05$. Exposure of mice to TMT increased FRA relative to saline or butyric acid treated mice. Again, the immediate test session produced the most robust increase in FRA. Analysis of variance of FRA cell counts in the prelimbic cortex of

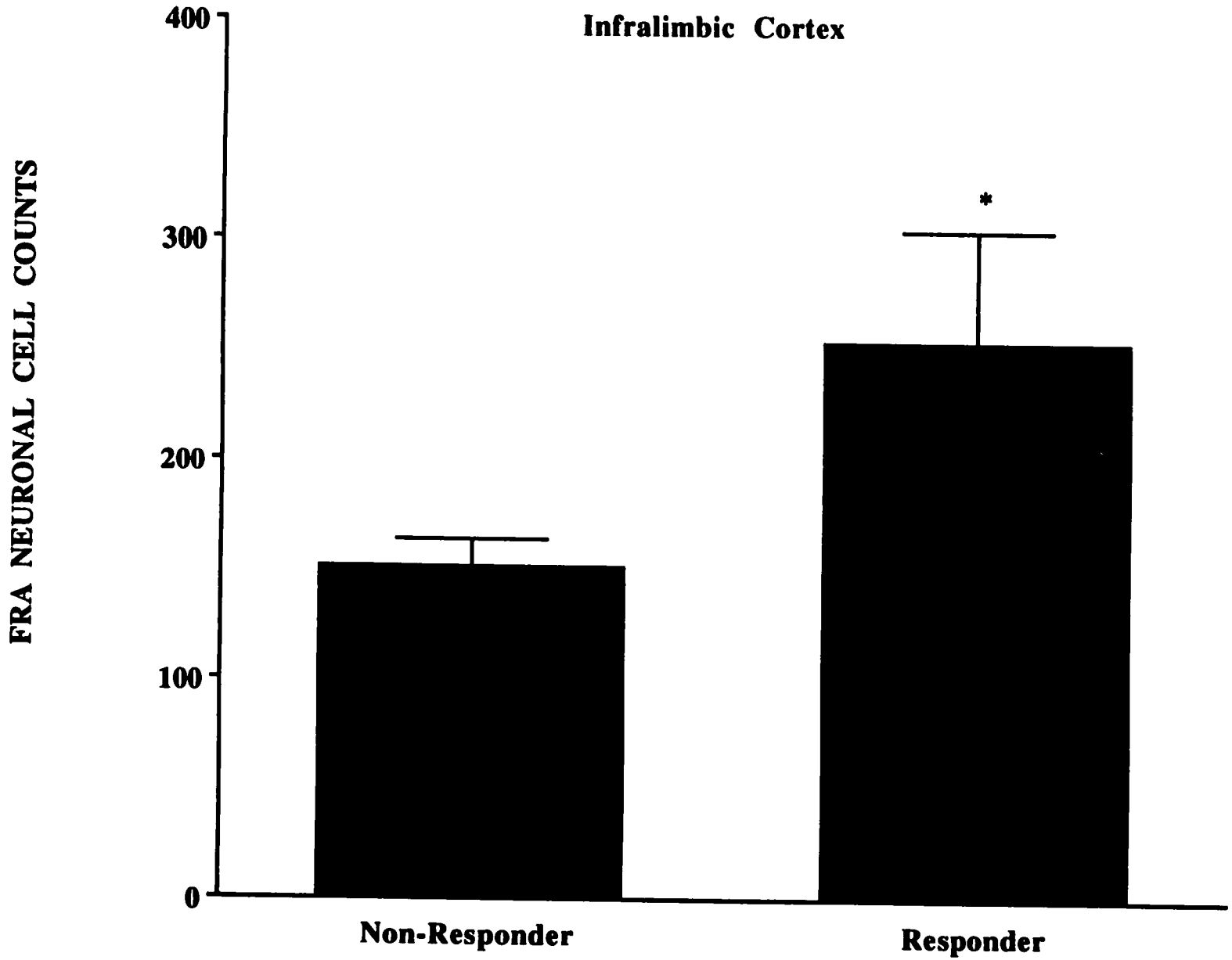
Figure 4.8: Cumulative (± S.E.M.) time in spent in the light chamber of the light dark box among mice classified as responders or non-responders to TMT based on freezing duration during original odor presentation in the home-cage immediately, 24 hours, 48 hours and 168 hours following odor exposure. Note: * depicts a statistically significant difference ($p < .05$) of freezing response to TMT on cumulative time spent in the light chamber of the light-dark box immediately following odor presentation.


CUMULATIVE TIME IN LIGHT CHAMBER
OF LIGHT DARK BOX (/600 SECS)
600
500
400
300
200
100
0
Non-Responder
Responder
*
Immediate
24 Hours
48 Hours
168 Hours
TEST SESSION

mice exposed to either 5 minutes or 10 minutes of saline or predator odor revealed a main effect of odor, $F(1,10)=13.43$, $p<.01$ and a main effect of time of odor exposure, $F(1,10)=7.07$, $p<.05$. Exposure of mice to either 5 minutes or 10 minutes of TMT increased FRA relative to saline treated mice. Furthermore, a 10 minute predator exposure increased FRA relative to 5 minute TMT exposure. Analysis of variance of FRA cell counts in the infralimbic cortex of mice exposed to either 5 minutes or 10 minutes of saline or predator odor failed to reveal any differences among treatment conditions (see Table 4.1). Analysis of variance of FRA cell counts in the prelimbic cortex of mice in the No LD condition termed responders or non-responders revealed a main effect of test session, $F(1,15)=3.82$, $p<.05$. Analysis of variance of FRA cell counts in the infralimbic cortex of mice in the No LD condition termed responders or non-responders failed to reveal any differences among treatment conditions (data not shown). Analysis of variance of FRA cell counts in the prelimbic and infralimbic cortices of mice termed anxious or non-anxious in the light-dark box irrespective of odor or test session failed to reveal any differences among treatment conditions. Analysis of variance of FRA cell counts in the infralimbic cortex of mice in the LD condition revealed a main effect of odor response $F(1, 18)=5.456$, $p<.05$. Responders demonstrated an increase in FRA relative to non-responders in the light-dark paradigm (See Figure 4.9).

Insert Figure 4.9 about here

Figure 4.9: Mean FRA cell counts (± S.E.M.) in the infralimbic cortex of mice in the light dark box (LD) condition termed responders or non-responders to fox odor based on freezing duration during original odor presentation in the home-cage. Note: FRA cell counts are collapsed over test sessions. The * depicts a statistically significant difference ($p < .05$) of freezing response to TMT on FRA expression in the infralimbic cortex.


Infralimbic Cortex
FRA NEURONAL CELL COUNTS
400
300
200
100
0
*
Non-Responder
Responder
HOME-CAGE FREEZING BEHAVIOR
IN RESPONSE TO TMT

GROUP	VTA	NAcCore	NAcShell	IFL	PRL	BLA	CEA	MEA
SAL IMM	11±5	367 ±58	292 ±50	115 ±2	651 ±31	98 ±20	113 ±17	72 ±13
SAL 24 H	19 ±8	218 ±30	171±28	131 ±11	349 ±52	112 ±21	155 ±25	86 ±16
SAL 48 H	40 ±6	257 ±46	218 ±63	119 ±23	295 ±58	76 ±15	161 ±33	92 ±19
SAL168H	30±10	358±62	218±41	96±12	250±24	97±14	128±16	97±15
BA IMM	20±3	486±37	445±64	144±11	486±36	55±11	121±17	58±10
BA 24 H	30±18	407±34	259±30	110±10	352±19	88±20	141±7	83±6
BA 48 H	36±12	267±60	158±42	110±12	274±46	94±23	125±18	90±21
BA 168 H	27±18	146±25	96±26	97±13	323±58	68±15	109±23	57±13
TMTIMM	60±14	875±94	719±35	196±46	674±131	113±17	162±29	75±9
TMT 24 H	15±5	420±41	269±26	144±27	445±70	79±14	119±12	63±18
TMT 48 H	40±9	308±67	242±44	185±52	391±34	86±11	167±19	95±13
TMT168H	32±7	228±50	270±97	169±25	366±13	59±9	110±23	60±14
SALIMM NO LD	19±4	288±23	343±43	141±24	383±54	119±46	159±14	60±17
SAL 24H NO LD	17±2	164±40	143±15	72±7	248±20	109±14	155±22	99±14
SAL 48H NO LD	15±2	188±55	150±25	107±18	315±46	83±13	113±14	68±15
SAL168H NO LD	15±5	183±35	174±26	99±14	213±21	113±11	149±14	84±10
BA IMM NO LD	26±7	486±37	445±64	157±16	493±38	132±30	155±47	88±22
BA 24 H NO LD	19 ±6	229±41	161±20	117±24	293±37	89±16	179±31	91±17
BA 48 H NO LD	32±6	261±72	173±22	113±15	301±33	116±25	164±28	56±17
BA 168 H NO LD	20±4	234±35	230±42	87±12	246±23	148±23	166±34	69±11
TMTIMM NO LD	82±8	692±56	657±101	185±36	597±79	112±27	155±23	64±4
TMT 24H NO LD	40±12	358±37	241±44	189±22	403±50	90±20	156±31	80±12
TMT 48H NO LD	42±11	409±30	366±89	168±23	452±30	129±26	189±48	94±29
TMT168H NO LD	34±19	255±45	173±30	134±25	369±18	88±23	125±20	79±19
5MINSAL	-	241±90	157±35	105±6	253±47	108±18	166±15	86±17
5MINTMT	-	419±115	273±96	166±32	438±41	129±39	182±16	114±26

TABLE 4.1: Immunohistological Analysis of FRA positive neuronal cell counts.
(SAL=Saline BA=Butyric Acid TMT=Predator odor NO LD= No light-dark box test VTA =ventral tegmental area NAcCore = core of the nucleus accumbens NAcShell= shell of the nucleus accumbens IFL = infralimbic cortex PFL= prelimbic cortex BLA = basolateral nucleus of the amygdala CEA = central nucleus of the amygdala MEA = medial nucleus of the amygdala)

Ventral Tegmental Area:

Analysis of variance of FRA cell counts from the dorsal aspects of the VTA among mice following odor exposure revealed a main effect of odor $F(2,89) = 13.316$, $p<.001$ and an odor x test session interaction $F(6,89) = 3.394$, $p<.01$. Exposure of mice to predator odor increased FRA in the immediate test session interval (See Table 4.1). Analysis of variance of FRA cell counts from the dorsal aspects of the VTA of mice in the LD and No LD conditions termed anxious/non-anxious or responders/non-responders failed to reveal any differences among treatment conditions(data not shown)

Nucleus Accumbens:

Analysis of variance of FRA cell counts from the core of the nucleus accumbens among mice following odor exposure revealed a main effect of odor $F(2,89) = 23.784$, $p<.001$, a main effect of light-dark box exposure $F(1,89) = 4.942$, $p<.05$, a main effect of test session $F(3,89) = 37.392$, $p<.001$ and an odor x test session interaction $F(6,89) = 4.903$, $p<.001$. Mice exposed to butyric acid or TMT displayed enhanced FRA relative to saline treated mice immediately post-stressor. Mice exposed to the light-dark box exhibited enhanced FRA relative to mice in the No LD condition. Analysis of variance of FRA cell counts from the shell of the nucleus accumbens among mice following odor exposure revealed a main effect of odor $F(2,89) = 19.855$, $p<.001$, a main effect of test session $F(3,89) = 40.687$, $p<.001$ and an odor x test session interaction $F(6,89) = 6.449$, $p<.001$. Mice exposed to butyric acid or TMT displayed enhanced FRA relative to saline treated mice immediately post-stressor. Mice exposed to the light-dark box exhibited enhanced FRA relative to mice in the No LD condition. Analysis of variance of FRA cell counts from the core and shell of the nucleus accumbens of mice exposed to either 5 minutes or 10 minutes of saline or predator odor

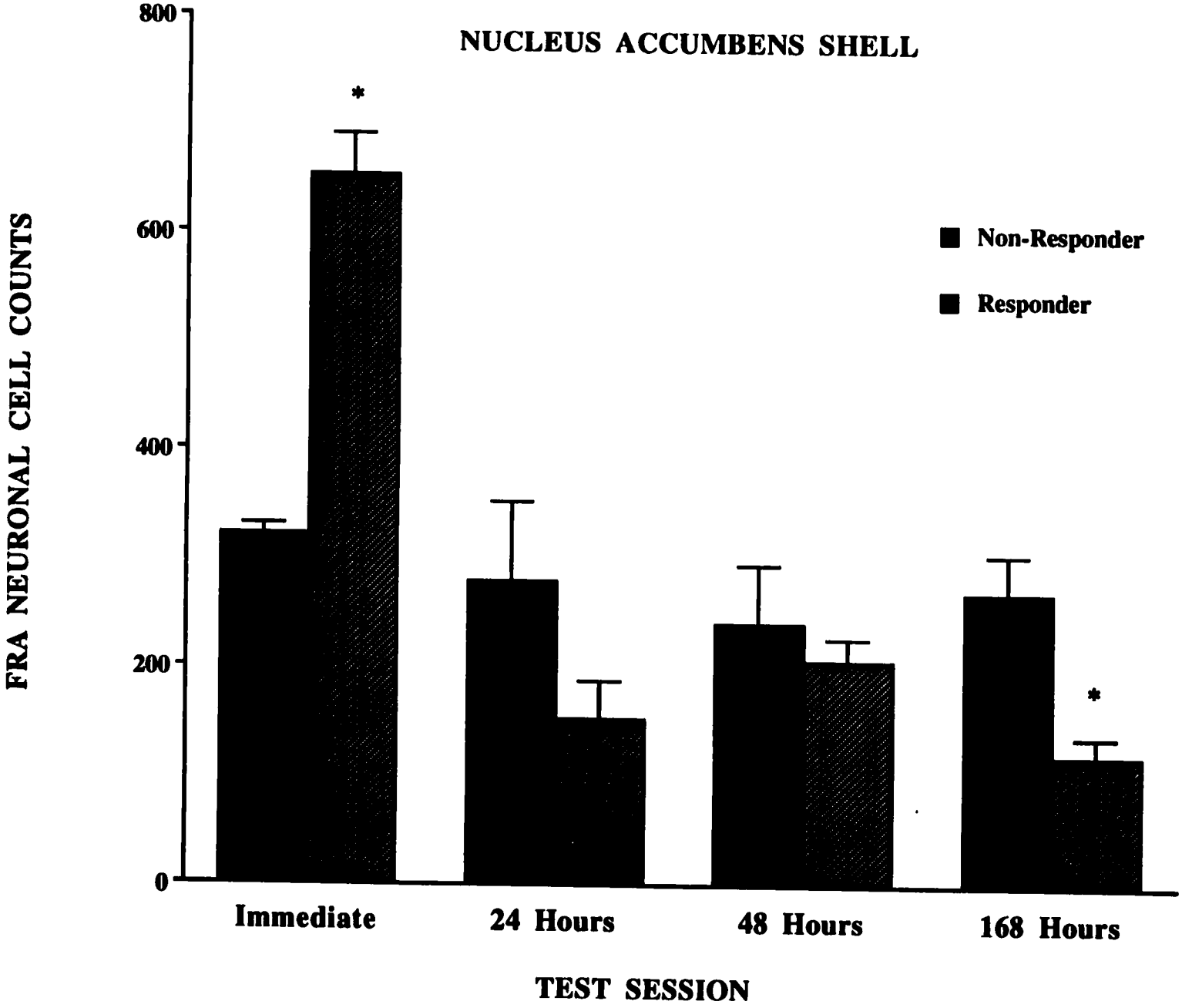
revealed a main effect of odor $F(1,12) = 15.120$, $p<.01$ and $F(1,12) = 6.907$, $p<.05$, respectively. Mice exposed to TMT displayed enhanced FRA relative to saline treated mice (See Table 4.1).

Analysis of variance of FRA cell counts in the core of the nucleus accumbens of mice in the No LD condition termed responders or non-responders to predator odor revealed a main effect of test session $F(3, 17) = 35.648$, $p<.001$. FRA immunoreactivity was greatest immediately post-stressor (data not shown). Analysis of variance of FRA cell counts in the shell of the nucleus accumbens of mice in the No LD condition termed responders or non-responders to predator odor revealed a main effect of test session $F(3, 17) = 16.888$, $p<.001$ and an odor response x test session interaction $F(2, 17) = 8.589$, $p<.01$. Responders displayed enhanced FRA relative to non-responders immediately followed by decreased FRA levels 168 hours post-stressor (See Figure 4.10).

Insert Figure 4.10 about here

Analysis of variance of FRA cell counts in the core of the nucleus accumbens of mice in the LD condition termed responders or non-responders to predator odor revealed a main effect of test session $F(3, 13) = 12.90$, $p<.001$, with the immediate test interval resulting in enhanced FRA relative to the other test intervals (data not shown). Analysis of variance of FRA cell counts in the shell of the nucleus accumbens of mice in the LD condition termed responders or non-responders to predator odor revealed a main effect of anxiety in the LD $F(1, 13) = 4.997$, $p<.05$ and a main effect of test session $F(3, 13) = 21.213$, $p<.001$. FRA was elevated on the immediate test session relative to 24 , 48 or 168 hours following odor

Figure 4.10: Mean FRA cell counts (± S.E.M.) in the shell of the nucleus accumbens of mice not tested in the light dark box (NO LD condition) termed responders or non-responders to fox odor based on freezing duration during original odor presentation in the home-cage immediately, 24 hours, 48 hours and 168 hours following odor exposure. Note: * depicts a statistically significant difference ($p < .05$) of freezing response to TMT on FRA expression in the shell of the nucleus accumbens.


NUCLEUS ACCUMBENS SHELL
FRA NEURONAL CELL COUNTS
800
600
400
200
0
*
*
Non-Responder
Responder
Immediate
24 Hours
48 Hours
168 Hours
TEST SESSION

presentation. Anxious mice displayed increased FRA in the shell of the nucleus accumbens relative to non-anxious mice (See Figure 4.11).

Insert Figure 4.11 about here

Amygdala:

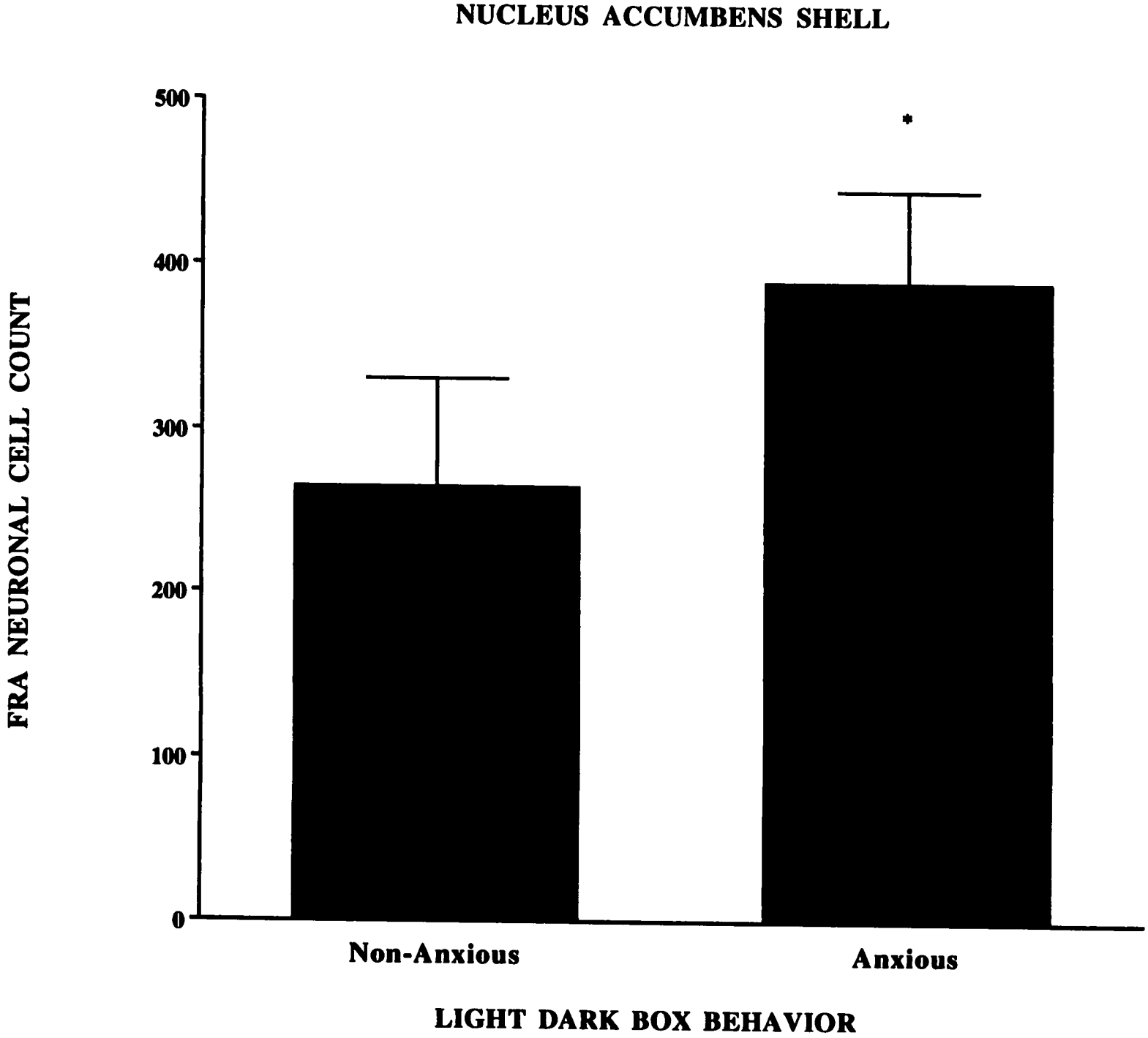
Analysis of variance of FRA cell counts from the central nucleus (CEA) and the basolateral nucleus (BLA) of the amygdala in CD-1 mice revealed a main effect of light-dark box exposure $F(1,93) = 3.998$, $p<.05$ and $F(1,92) = 10.244$, $p<.01$, respectively. Mice exposed to the light-dark box exhibited reduced FRA relative to mice in the No LD condition. Analysis of variance of FRA cell counts from the CEA of mice exposed to either 5 minutes or 10 minutes of saline or predator odor revealed a main effect of odor exposure time $F(1,11) = 6.18$, $p<.05$. Among CD-1 mice, a 10 minute odor exposure reduced FRA relative to 5 minute exposure. Analysis of variance of FRA cell counts from all other sub-regions of the amygdala of mice exposed to either 5 minutes or 10 minutes of saline or predator odor failed to reveal any significant differences among treatment conditions (See Table 4.1). Analysis of variance of FRA cell counts from sub-regions of the amygdala of mice in the No LD condition termed responders or non-responders to predator odor or in the LD condition termed anxious or non-anxious failed to reveal any differences among treatment conditions (data not shown).

Enkephalin Neuronal Cell Counts

Nucleus Accumbens

Analysis of variance of ENK cell counts from the core of the nucleus accumbens in mice revealed a main effect of light-dark box exposure $F(1,46) = 9.204$, $p<.01$ and a main effect

Figure 4.11: Mean FRA cell counts (± S.E.M.) in the shell of the nucleus accumbens associated with anxiety in the light dark box. Note: * depicts a statistically significant difference ($p < .05$) of light dark box anxiety following TMT exposure on FRA expression in the shell of the nucleus accumbens.


NUCLEUS ACCUMBENS SHELL
FRA NEURONAL CELL COUNT
500
400
300
200
100
0
*
Non-Anxious
Anxious
LIGHT DARK BOX BEHAVIOR

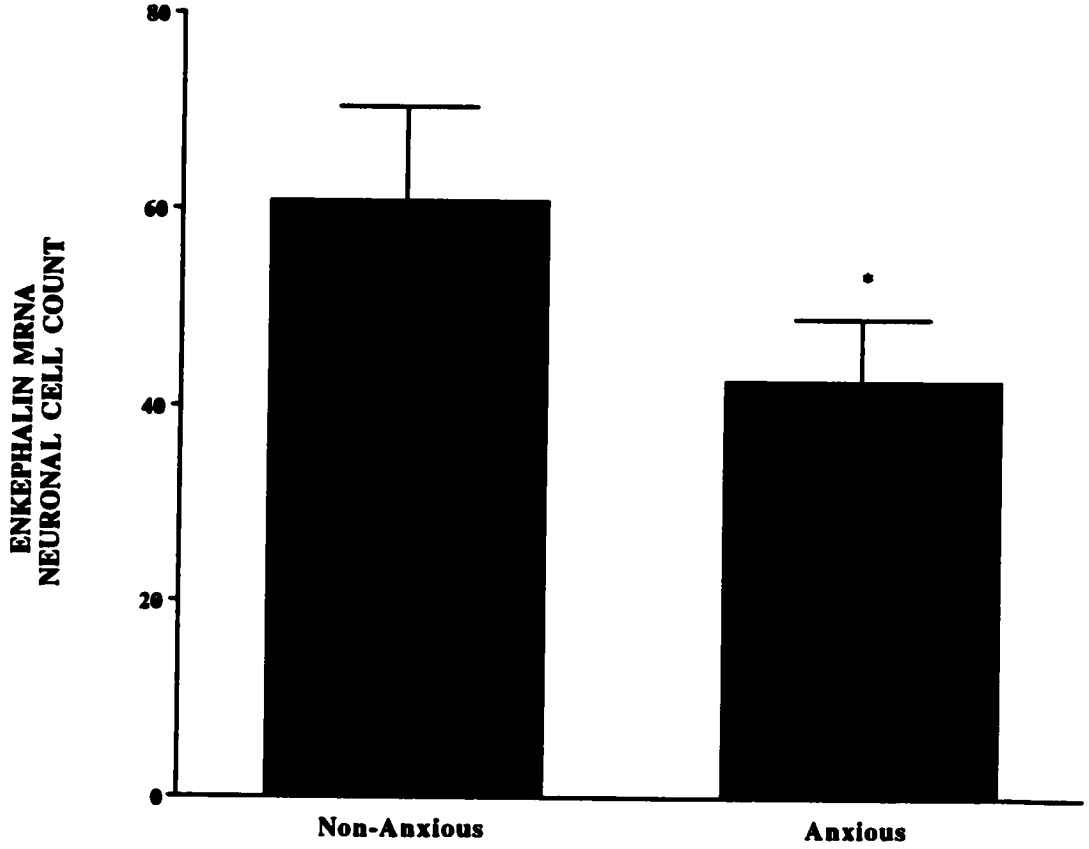
of test session F(3,46) =2.754, p<.05. Exposure of mice to the light-dark apparatus resulted in increased ENK. The greatest increase in ENK mRNA among mice following odor exposure was 24 hours post-stressor. Analysis of variance of ENK cell counts from the shell of the nucleus accumbens among mice following odor exposure failed to reveal any significant differences among treatment conditions (F's < 1). Analysis of variance of ENK cell counts from the core of the nucleus accumbens of mice exposed to either 5 minutes or 10 minutes of saline or predator odor revealed a main effect of exposure time F(1,11) =7.264, p<.05. A 10 minute exposure to either of the odorant conditions decreased ENK in mice relative to a 5 minute exposure. Analysis of variance of ENK cell counts from the shell of the nucleus accumbens among mice following odor exposure failed to reveal any significant differences among treatment conditions (F's<1; See Table 4.2).

Analysis of variance of ENK cell counts in the shell of the nucleus accumbens of mice in the No LD condition termed responders or non-responders to predator odor revealed a main effect of test session F(3, 8) =19.908, p<.05. ENK was enhanced in the immediate and 24 hour test intervals among mice following odor exposure relative to the 48 and 168 hour sessions (data not shown). Analysis of variance of ENK cell counts in the core of the nucleus accumbens of mice in the LD condition termed responders or non-responders to predator odor revealed a main effect of light-dark box anxiety F(1,8) =18.749, p<.01, odor response F(1, 8) =9.858, p<.01 and test session F(1, 8) =7.144, p<.01. Light-dark box anxiety decreased while freezing increased ENK in mice relative to non-anxious and non-freezing mice exposed to TMT, respectively (See Figure 4.12). ----------

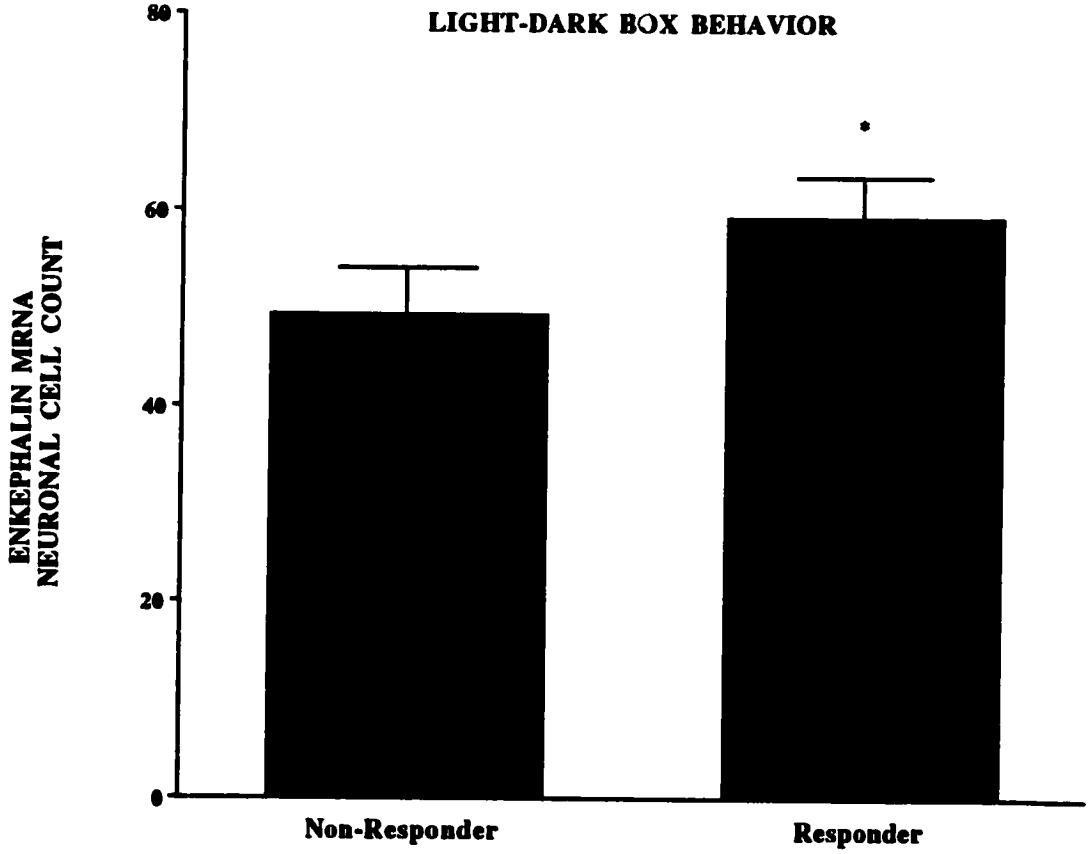
Insert Figure 4.12 about here

Figure 4.12: Mean ENK cell counts (± S.E.M.) in the core of the nucleus accumbens associated with anxiety in the light dark box (top panel) and in mice termed responders or non-responders to fox odor based on freezing duration during original odor presentation in the home-cage (bottom panel). Note: * depicts a statistically significant difference ($p < .05$) of light dark box anxiety or odor response following TMT exposure on ENK mRNA expression in the core of the nucleus accumbens.

NUCLEUS ACCUMBENS CORE



LIGHT-DARK BOX BEHAVIOR



HOME CAGE FREEZING BEHAVIOR
IN RESPONSE TO TMT

Amygdala:

Analysis of variance of ENK cell counts from the CEA and the BLA among mice following odor exposure revealed a main effect of test session $F(3,93) = 2.567$, $p<.05$ and $F(3,92) = 2.61$, $p<.05$, respectively. ENK was enhanced in the immediate and 24 hour test intervals relative to the 48 and 168 hour sessions. Analysis of variance of ENK cell counts from other sub-regions of the amygdala failed to reveal any significant differences among treatment conditions (data not shown). Analysis of variance of ENK cell counts from the BLA of mice exposed to either 5 minutes or 10 minutes of saline or predator odor revealed a main effect of odor exposure time $F(1,11) = 4.937$, $p<.05$. A 10 minute exposure to either of the odorant conditions decreased ENK relative to a 5 minute exposure. Analysis of variance of FRA cell counts from all other sub-regions of the amygdala of mice exposed to either 5 minutes or 10 minutes of saline or predator odor failed to reveal any significant differences among treatment conditions (See Table 4.2).

Analysis of variance of ENK cell counts in the CEA of mice in the No LD condition termed responders or non-responders to predator odor revealed a main effect of odor response $F(1, 12) = 5.377$, $p<.05$. Responders displayed decreased ENK in the CEA relative to non-responders (See Figure 4.13).

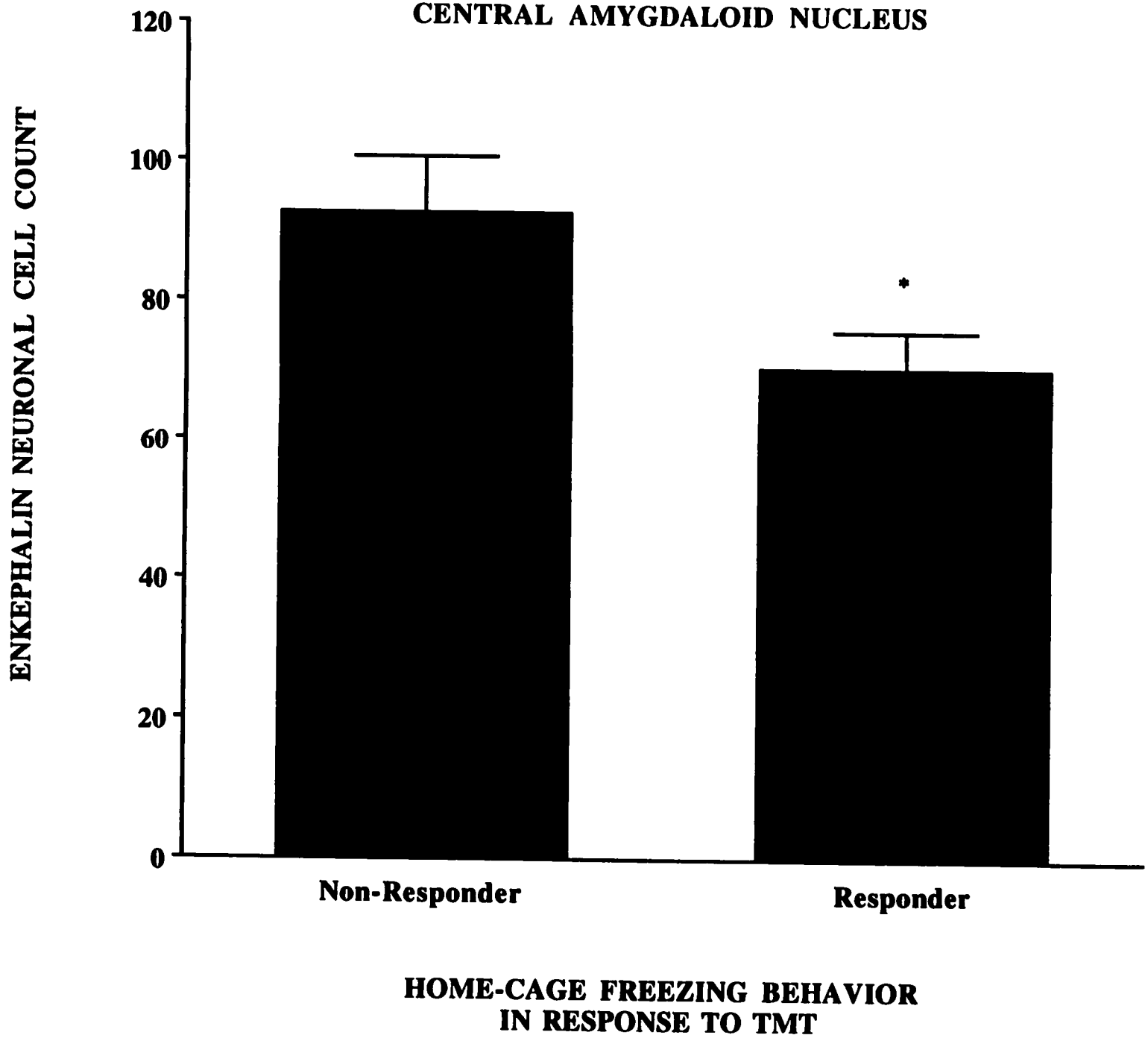
Insert Figure 4.13 about here

Analysis of variance of ENK cell counts from the BLA and the MEA of mice in the No LD condition termed responders or non-responders to predator odor failed to reveal any

GROUP	NAcCore	NAcShell	BLA	CEA	MEA
SAL IMMEDIATE	22±3	26±8	33±8	90±12	34±12
SAL 24 HOURS	82±18	80±18	43±6	99±12	36±9
SAL 48 HOURS	32±11	33±10	16±5	59±9	33±10
SAL 168 HOURS	18±5	15±7	12±4	65±15	29±13
BA IMMEDIATE	55±15	58±14	26±8	82±13	33±11
BA 24 HOURS	55±15	58±14	31±9	90±8	39±5
BA 48 HOURS	30±14	13±1	36±9	105±7	42±11
BA 168 HOURS	71±19	42±4	13±3.5	50±10	14±4
TMT IMMEDIATE	66±13	80±10	38±3	103±12	30±6
TMT 24 HOURS	45±12	41±12	27±8	96±9	34±17
TMT 48 HOURS	40±6	27±6	29±9	89±15	38±11
TMT168 HOURS	42±14	42±19	13±4	67±3	20±5
SAL IMMEDIATE NO LD	11±1	15±1	28±3	99±2	30±6
SAL 24 HOURS NO LD	11±6	25±22	48±12	103±24	52±14
SAL 48 HOURS NO LD	44±18	55±22	40±7	74±11	34±10
SAL 168 HOURS NO LD	24±12	31±17	34±7	94±13	27±4
BA IMMEDIATE NO LD	40±20	31±16	41±3	90±2	30±3
BA 24 HOURS NO LD	40±20	31±15	25±6	78±14	26±8
BA 48 HOURS NO LD	41±22	34±15	23±7	75±14	19±5
BA 168 HOURS NO LD	50±25	25±9	40±11	93±23	40±10
TMT IMMEDIATE NO LD	46±13	60±10	26±9	93±15	24±7
TMT 24 HOURS NO LD	87±16	70±14	31±9	70±15	29±7
TMT 48 HOURS NO LD	27±3	36±22	26±10	77±17	26±7
TMT168 HOURS NO LD	69±47	58±44	24±4	72±8	27±3
5 MIN SAL	43±9	36±7	42±7	91±16	28±6
5 MIN TMT	88±21	55±15	45±7	87±14	29±8

TABLE 4.2: Immunohistological Analysis of ENK positive neuronal cell counts. (SAL=Saline BA=Butyric Acid TMT=Predator odor NO LD= No light-dark box test). NAcCore = core of the nucleus accumbens NAcShell= shell of the nucleus accumbens BLA = basolateral nucleus of the amygdala CEA = central nucleus of the amygdala MEA = medial nucleus of the amygdala)

Figure 4.13: Mean ENK cell counts (± S.E.M.) in the central amygdaloid nucleus (CEA) of mice in the NO LD condition termed responders or non-responders to fox odor based on freezing duration during original odor presentation in the home-cage. ENK cell counts are collapsed over test sessions. Note: * depicts a statistically significant difference ($p < .05$) of odor response following TMT exposure on ENK mRNA expression in the CEA.


CENTRAL AMYGDALOID NUCLEUS
ENKEPHALIN NEURONAL CELL COUNT
120
100
80
60
40
20
0
*
Non-Responder
Responder
HOME-CAGE FREEZING BEHAVIOR
IN RESPONSE TO TMT

differences among treatment conditions. Analysis of variance of ENK cell counts in the BLA, CEA and MEA of mice in the LD condition termed responders or non-responders to predator odor revealed a main effect of light-dark box anxiety $F(1,15) = 4.575$, $p<.05$, $F(1,15) = 10.329$, $p<.01$ and $F(1,15) = 6.853$, $p<.05$. respectively. Anxious mice displayed increased ENK in the BLA, CEA and MEA relative to their non-anxious counterparts (See Figure 4.14).

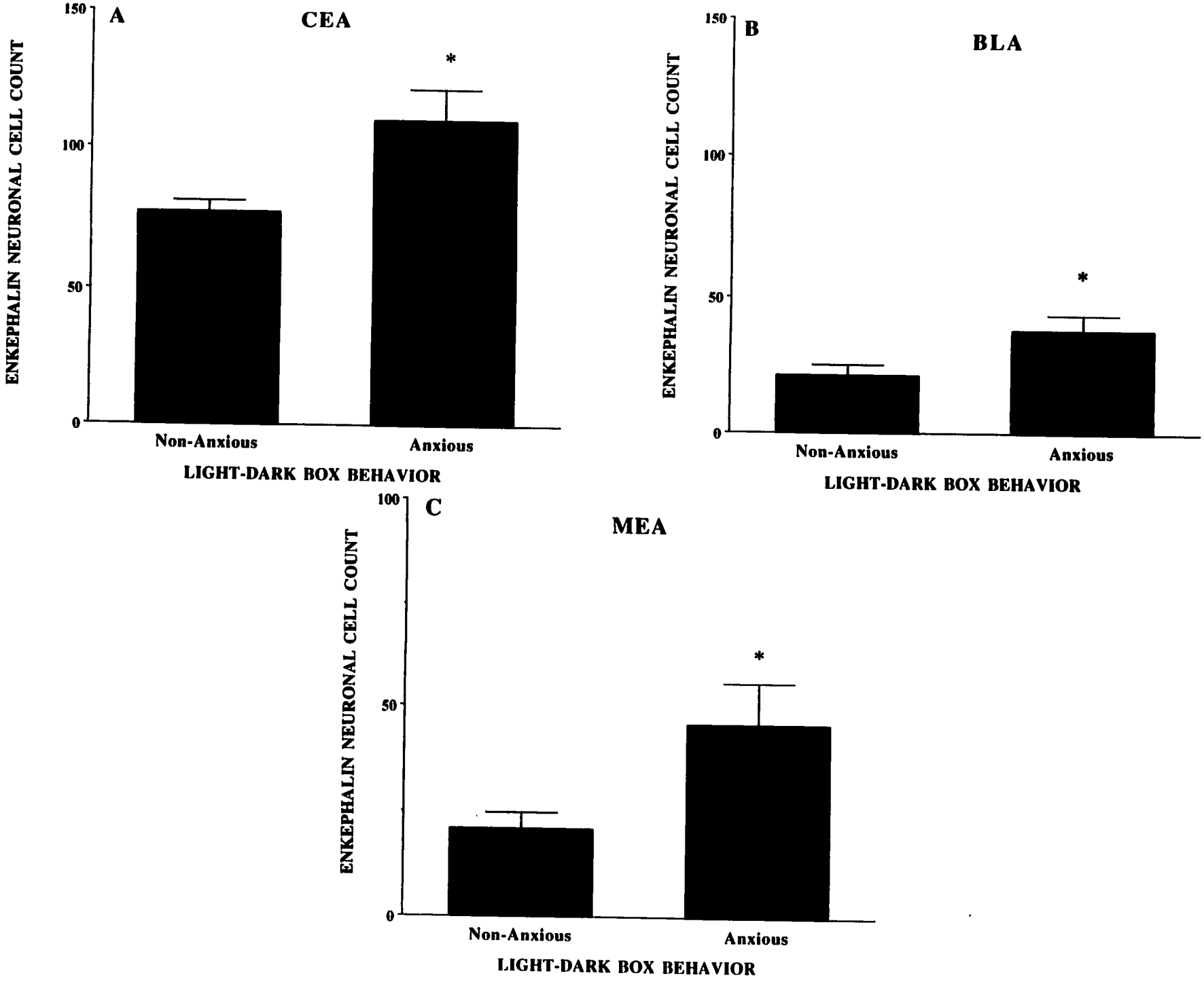
Insert Figure 4.14 about here

Double Labeled Enkephalin and FRA

Nucleus Accumbens:

Analysis of variance of double staining of ENK and FRA cell counts from the core of the nucleus accumbens among mice following odor exposure revealed a main effect of odor $F(2,46) = 7.083$, $p<.01$, a main effect of test session $F(3,46) = 10.102$, $p<.001$ and an odor x test session interaction $(6,46) = 4.029$, $p<.01$. Exposure of CD-1 mice to either butyric acid or TMT increased double labeled cell counts immediately following odor exposure. Analysis of variance of double labeled ENK and FRA neurons from the shell of the nucleus accumbens in mice also revealed a main effect of odor $F(2,46) = 4.713$, $p<.01$, a main effect of test session $F(3,46) = 10.734$, $p<.001$ and an odor x test session interaction $F(6,46)=3.195$, $p<.01$. The pattern of double labeled cell counts in the shell paralleled those exhibited in the core of the accumbens. Analysis of variance of double staining of ENK and FRA cell counts from the core of the nucleus accumbens failed to reveal any significant differences among treatment conditions ($F's<1$). Analysis of variance of double staining of ENK and FRA cell counts from the shell of the nucleus accumbens of mice exposed to either 5 minutes or 10 minutes of saline

Figure 4.14: Mean ENK cell counts (± S.E.M.) in the (A) central (CEA), (B) basolateral (BLA) and (C) medial (MEA) amygdaloid nuclei in mice classified as anxious in the light dark box following exposure to fox odor. ENK cell counts are collapsed over test sessions. Note: * depicts a statistically significant difference ($p < .05$) of light dark box anxiety following TMT exposure on ENK mRNA expression in the amygdaloid complex.


A
CEA
ENKEPHALIN NEURONAL CELL COUNT
150
100
50
0
*
Non-Anxious
Anxious
LIGHT-DARK BOX BEHAVIOR
B
BLA
ENKEPHALIN NEURONAL CELL COUNT
150
100
50
0
*
Non-Anxious
Anxious
LIGHT-DARK BOX BEHAVIOR
C
MEA
ENKEPHALIN NEURONAL CELL COUNT
100
50
0
*
Non-Anxious
Anxious
LIGHT-DARK BOX BEHAVIOR

or predator odor revealed a main effect of odor $F(1,11) = 8.407$, $p<.05$, a main effect of exposure time $F(1,11) = 6.882$, $p<.05$ and an odor x exposure time interaction $F(1,11) = 6.564$, $p<.05$. Mice exposed to 10 minutes of TMT displayed enhanced double immunoreactivity relative to both saline treated mice or mice merely exposed to 5 minutes of TMT (See Table 4.3).

Analysis of variance of double ENK and FRA cell counts in the shell of the nucleus accumbens of mice in the No LD condition termed responders or non-responders to predator odor revealed a main effect of odor response $F(1, 8) = 15.125$, $p<.05$ and test session $F(3, 8) = 57.195$, $p<.01$. Responders displayed enhanced ENK-FRA counts with the immediate test session hosting the most prominent changes (See Figures 4.15 and 4.16).

Insert Figures 4.15 and 4.16 about here

Analysis of variance of double ENK and FRA cell counts in the core of the nucleus accumbens of mice in the No LD condition termed responders or non-responders to predator odor failed to reveal any differences among treatment conditions (data not shown). Analysis of variance of double ENK and FRA cell counts in the core of the nucleus accumbens of mice in the LD condition termed responders or non-responders to predator odor revealed a main effect of test session $F(1, 8) = 5.245$, $p<.05$. Again, the immediate test interval revealed the most robust ENK-FRA staining (data not shown). Analysis of variance of double ENK and FRA cell counts in the shell of the nucleus accumbens of mice in the LD condition termed responders or non-responders to predator odor failed to reveal any differences among treatment conditions (data not shown). Analysis of variance of double labeled ENK and FRA

GROUP	NAcCore	NAcShell	BLA	CEA	MEA
SAL IMMEDIATE	12±4	11±7	15±4	29±4	15±6
SAL 24 HOURS	23±7	13±3	22±5	42±8	13±5
SAL 48 HOURS	9±2	10±6	5±1	21±5	19±8
SAL 168 HOURS	9±1	2±1	6±2	20±7	10±4
BA IMMEDIATE	27±17	11±5	15±8	32±7	18±7
BA 24 HOURS	14±4	9±3	23±9	42±7	27±6
BA 48 HOURS	6±1	3±1	12±3	40±7	15±5
BA 168 HOURS	8±5	1±1	3±1	13±4	2±1
TMT IMMEDIATE	60±26	37±9	12±2	42±9	10±2
TMT 24 HOURS	17±3	14±3	10±3	35±7	9±3
TMT 48 HOURS	19±14	4±2	20±9	52±24	23±13
TMT168 HOURS	16±12	24±19	5±2	15±2	5±1
SAL IMMEDIATE NO LD	13±1	10±1	9±4	46±1	11±6
SAL 24 HOURS NO LD	8±8	3±3	28±10	42±14	25±8
SAL 48 HOURS NO LD	24±16	7±3	15±4	22±4	12±5
SAL 168 HOURS NO LD	11±7	6±4	15±5	33±7	8±2
BA IMMEDIATE NO LD	68±24	52±14	14±5	29±5	10±2
BA 24 HOURS NO LD	17±6	11±3	9±3	29±9	9±3
BA 48 HOURS NO LD	11±8	7±3.	8±3	29±6	3±2
BA 168 HOURS NO LD	16±8	3±1	20±11	32±12	18±9
TMT IMMEDIATE NO LD	66±14	54±18	8±3	37±8	7±2
TMT 24 HOURS NO LD	32±18	10±4	13±5	26±8	13±5
TMT 48 HOURS NO LD	4±2	8±1	11±5	33±11	10±6
TMT168 HOURS NO LD	15±9	5±2	10±3	30±3	10±4
5 MIN SAL	12±3	10±4	19±7	40±12	10±3
5 MIN TMT	47±29	12±7	20±6	43±8	23±7

TABLE 4.3: Immunohistological Analysis of FRA/ENK positive neuronal cell counts. (SAL=Saline BA=Butyric Acid TMT=Predator odor NO LD= No light-dark box test). NAcCore = core of the nucleu accumbens NAcShell= shell of the nucleus accumbens BLA = basolateral nucleus of the amygdala CEA = central nucleus of the amygdala MEA = medial nucleus of the amygdala)

Figure 4.15: Mean double FRA/ENK cell counts (± S.E.M.) in the shell of the nucleus accumbens of mice in the NO LD condition termed responders or non-responders to fox odor based on freezing duration during original odor presentation in the home-cage. Note: * depicts a statistically significant difference ($p < .05$) of odor response following TMT exposure on double FRA/ENK mRNA expression in the shell of the nucleus accumbens.

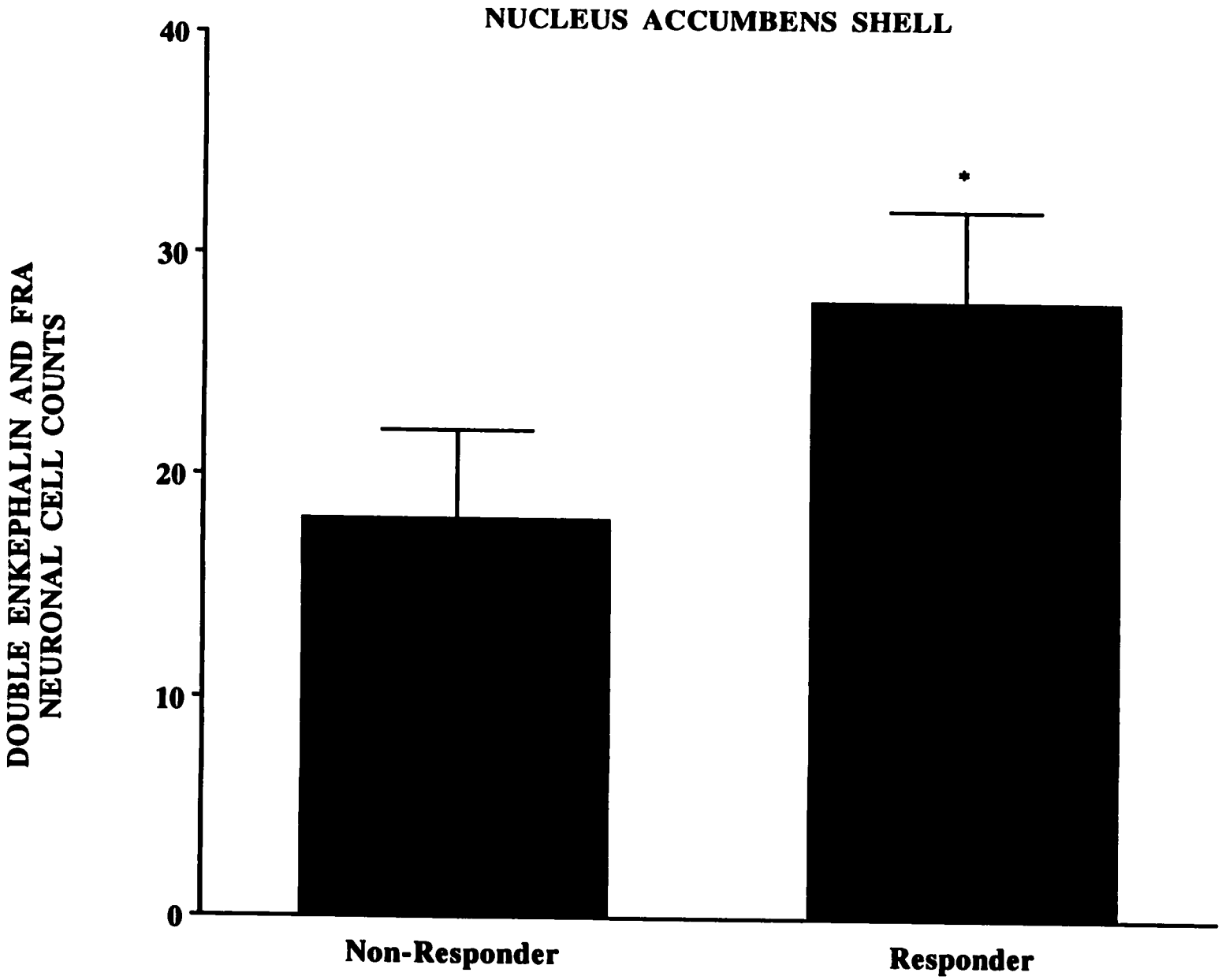
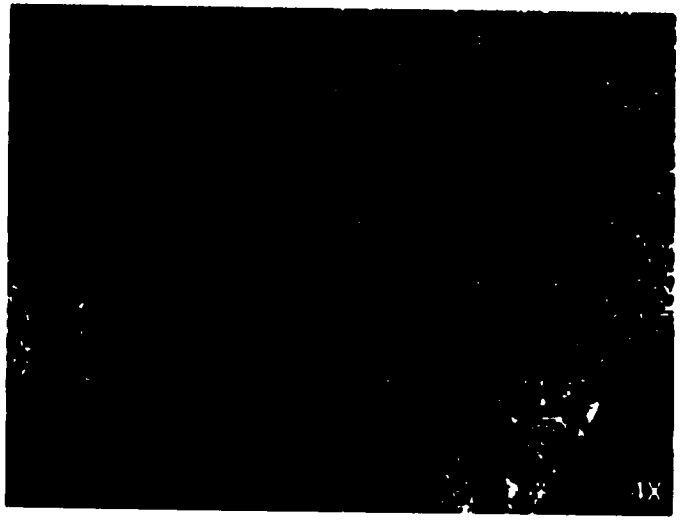
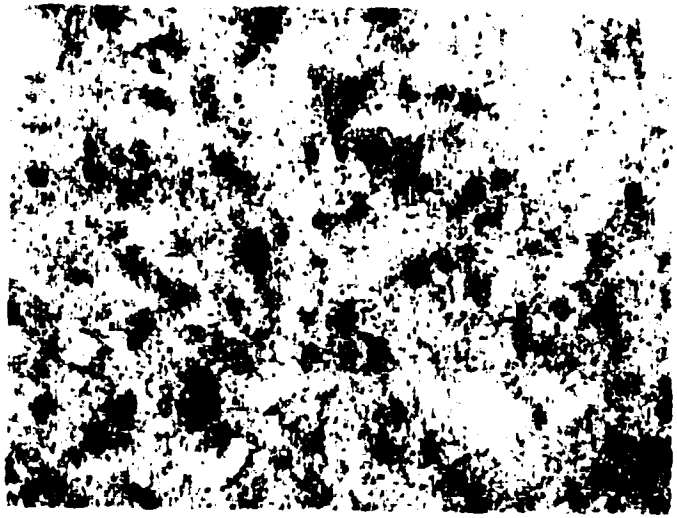

NUCLEUS ACCUMBENS SHELL
DOUBLE ENKEPHALIN AND FRA NEURONAL CELL COUNTS
40
30
20
10
0
*
Non-Responder
Responder
HOME-CAGE FREEZING BEHAVIOR IN RESPONSE TO TMT

Figure 4.16: Photomicrograph (4X; dark field and light field, 100X and 400X) of the nucleus accumbens core and shell depicting FRA (brown), ENK (black) and double FRA/ENK (brown and black) neuronal staining following either saline, butyric acid or TMT exposure among mice not exposed to the light dark box. aco= anterior commissure CP=caudate putamen

Nucleus Accumbens
no Light Dark
TMT exposure





neurons in the core and shell of the nucleus accumbens of mice in the LD condition termed anxious or non-anxious failed to reveal any differences among treatment conditions (data not shown).

Amygdala:

Analysis of variance of double staining of ENK and FRA cell counts from the BLA and CEA among mice following odor exposure revealed a main effect of test session $F(3,91) = 3.016$, $p<.05$ and $F(3,92) = 2.941$, $p<.05$, respectively. The immediate and 24 hour test interval revealed the most robust ENK-FRA staining. Analysis of variance of double staining of ENK and FRA cell counts from the CEA revealed a test session x light-dark box interaction $F(3,92) = 2.694$, $p<.05$. ENK-FRA counts were reduced in mice in the LD condition 168 hours post-odor exposure. Analysis of variance of double staining of ENK and FRA cell counts from other sub-regions of the amygdala failed to reveal any significant differences among treatment conditions (data not shown). Analysis of variance of double labeled ENK and FRA cells from all sub-regions of the amygdala examined among mice exposed to either 5 minutes or 10 minutes of saline or predator odor failed to reveal any significant differences among treatment conditions (See Table 4.3).

Analysis of variance of double labeled ENK and FRA cells in the CEA of mice in the No LD condition termed responders or non-responders to predator odor revealed a main effect of test session $F(3, 12) = 3.353$, $p<.05$ (data not shown). Analysis of variance of double labeled ENK and FRA cells from all other sub-regions of the amygdala examined among mice in the No LD condition termed responders or non-responders to predator odor failed to reveal any differences among treatment conditions (data not shown). Analysis of variance of double labeled ENK and FRA cells in the BLA, and CEA of mice in the LD condition termed

responders or non-responders to predator odor revealed a main effect of light-dark box anxiety $F(1,15) = 4.184$, $p<.05$ and $F(1,15) = 4.166$, $p<.05$, respectively. Anxious mice displayed increased double ENK and FRA in the BLA and CEA relative to their non-anxious counterparts (See Figures 4.17, 4.18 and 4.19).

Insert Figures 4.17, 4.18 and 4.19 about here

Densitometric Analysis of ENK mRNA

Nucleus Accumbens:

Analysis of variance of the optical density of ENK mRNA from the core and shell of the nucleus accumbens failed to reveal any differences among treatment conditions (data not shown). Analysis of variance of the optical density of ENK in the core and shell of the nucleus accumbens of mice in the No LD condition termed responders or non-responders to predator odor revealed a main effect of test session $F(3, 7) = 8.606$, $p<.01$ and $F(3,7) = 5.272$, $p<.05$, respectively. ENK mRNA values were lowest following the 48 hour test session from both the core and shell of the accumbens (See Table 4.4).

Amygdala:

Basolateral Nucleus of the Amygdala

Analysis of variance of the integrated density of ENK from the BLA failed to reveal any differences among treatment conditions (see Table 4.4). However, analysis of variance of the integrated density of ENK in the BLA of mice in the LD condition termed anxious or non-anxious/responders or non-responders to predator odor revealed a main effect of light-

Figure 4.17: Photomicrograph (4X; dark field and light field, 100X and 400X) of the amygdaloid complex depicting FRA (brown), ENK (black) and double FRA/ENK (brown and black) neuronal staining following saline, butyric acid or TMT exposure among mice not exposed to the light dark box.

Amygdaloid Complex
no Light Dark
TMT exposure

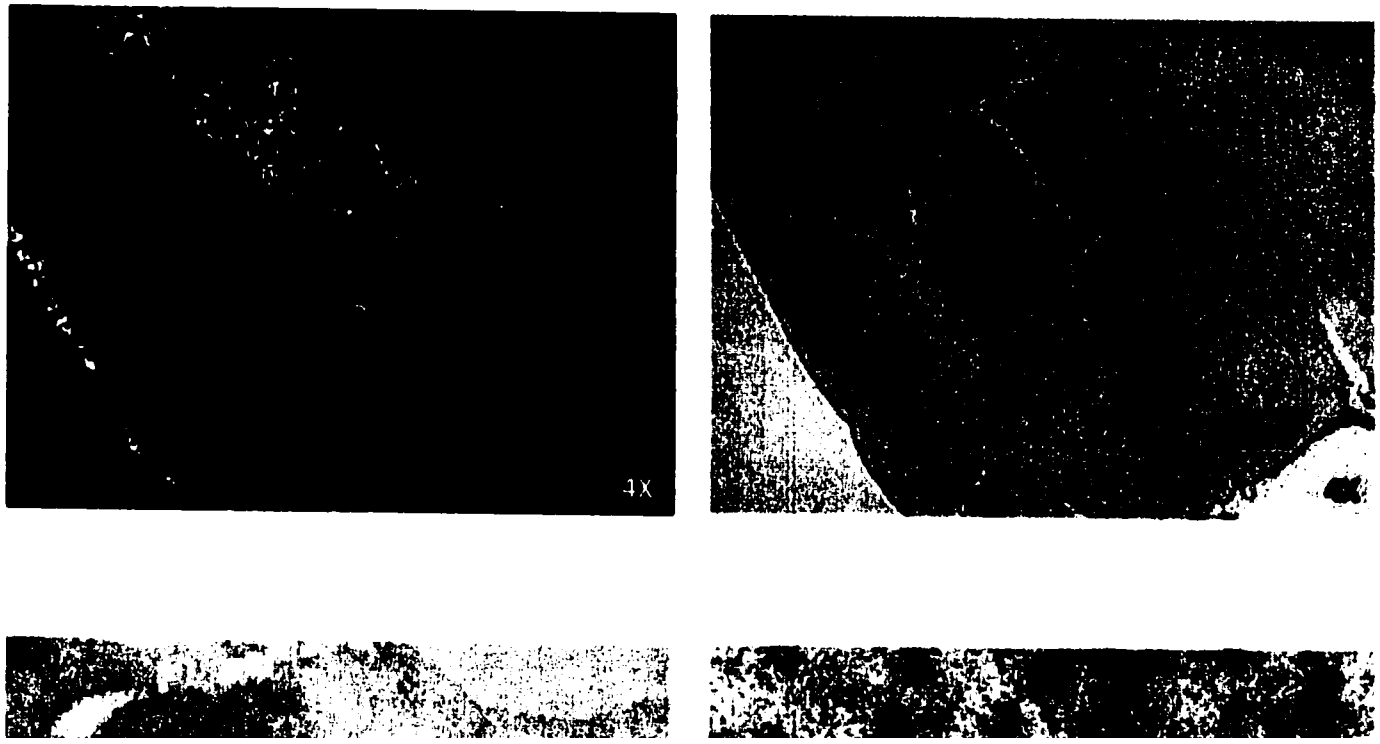


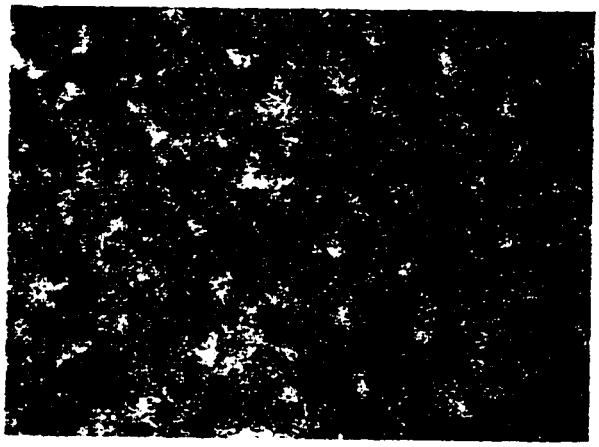
Figure 4.18: Photomicrograph (400X) of the amygdaloid complex (right) and nucleus accumbens (left) depicting FRA (brown), ENK (black) and double FRA/ENK neuronal staining following saline, butyric acid or TMT exposure among mice tested in the light dark box.

Amygdaloid Complex

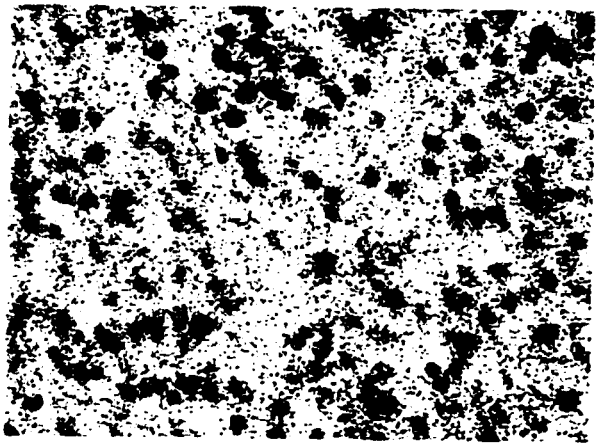
Nucleus Accumbens

Light-Dark paradigm

SA



BA



TMT

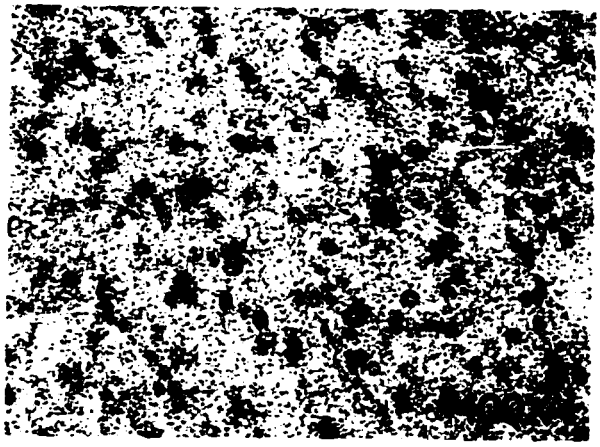
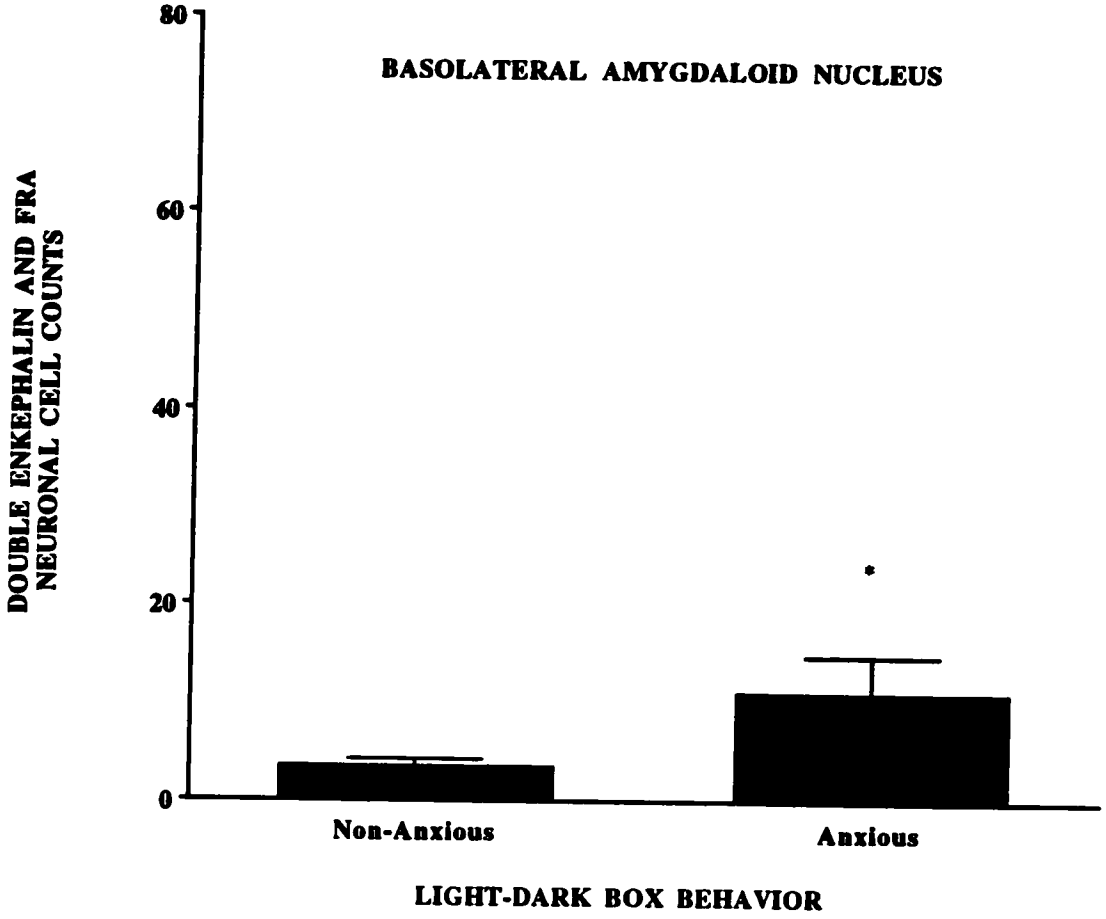
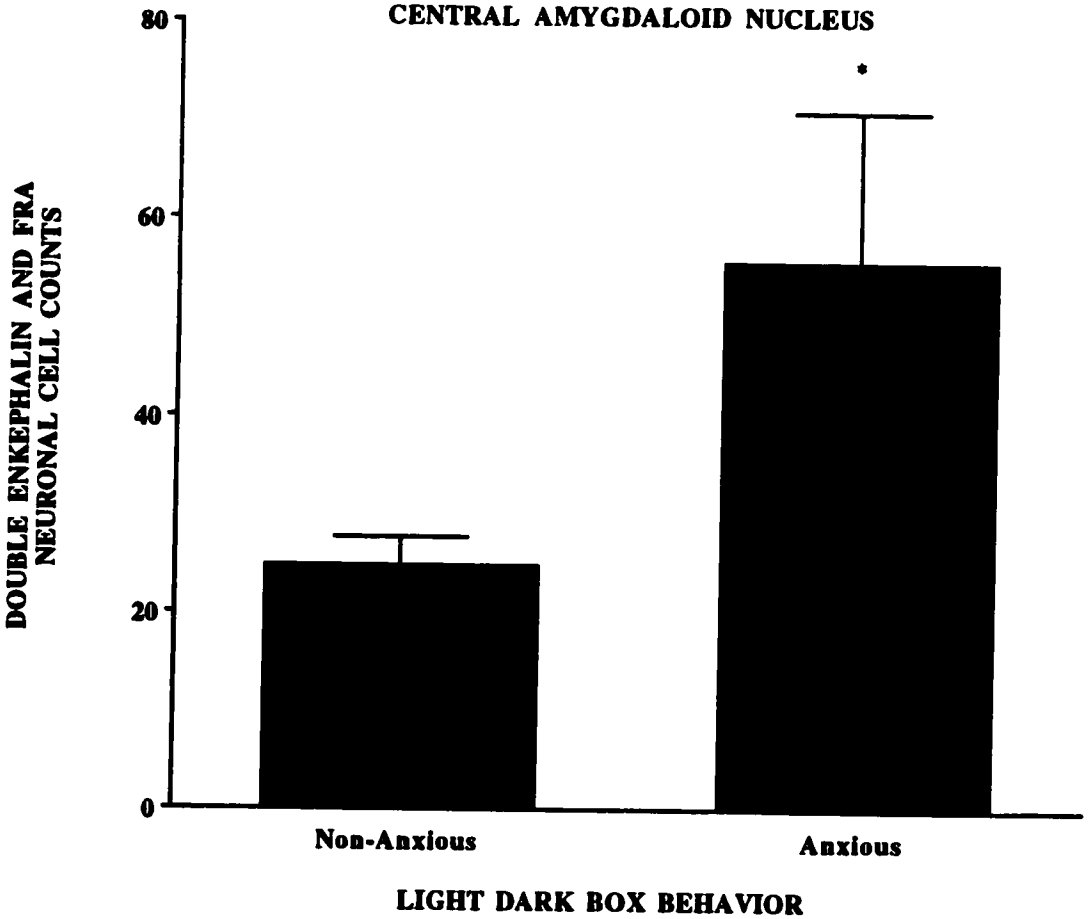


Figure 4.19: Mean double FRA/ENK cell counts (± S.E.M.) in the basolateral (BLA) (top panel) and central (CEA) (bottom panel) amygdaloid nuclei in mice classified as anxious or non anxious in the light dark box following exposure to fox odor. FRA/ENK cell counts are collapsed over test sessions. Note: * depicts a statistically significant difference ($p < .05$) of light dark box anxiety following TMT exposure on double FRA/ENK mRNA expression in the amygdaloid complex.


BASOLATERAL AMYGDALOID NUCLEUS
DOUBLE ENKEPHALIN AND FRA NEURONAL CELL COUNTS
80
60
40
20
0
*
Non-Anxious
Anxious
LIGHT-DARK BOX BEHAVIOR


CENTRAL AMYGDALOID NUCLEUS
DOUBLE ENKEPHALIN AND FRA NEURONAL CELL COUNTS
80
60
40
20
0
*
Non-Anxious
Anxious
LIGHT DARK BOX BEHAVIOR

GROUP	BLADI	CEADI	MEADI	DOCORE	DOSHELL
SAL IMM	1248.1 ±480.2	2887.4 ±443.7	241.4 ±66.7	1.42±0.35	1.19±0.21
SAL 24 H	794.7 ±171.5	3040.1 ±524.2	131.6 ±19.1	1.85±0.39	1.74±0.36
SAL 48 H	728.2 ±256.2	2051.3 ±601.6	138.9 ±31.7	1.4±0.16	1.29±0.24
SAL168 H	848.1 ±292.9	3348.6 ±1287.8	153.1 ±26.5	1.09±0.17	1.03±0.21
BA IMM	858.4 ±204.6	2656.2 ±326.4	186.0 ±28.5	1.62±0.25	1.8±0.41
BA 24 H	1023.5 ±189.2	2357.5 ±694.4	223.0 ±83.8	1.65±0.26	1.65±0.29
BA 48 H	505.0 ±101.1	3186.3 ±331.3	127.0 ±19.1	1.16±0.13	1.16±0.23
BA 168 H	518.7 ±95.9	2085.1 ±518.5	122.1 ±11.2	0.98±0.23	1.22±0.18
TMTIMM	882.2 ±197.9	2393.4 ±554.0	151.6 ±40.4	1.42±0.22	1.5±0.21
TMT 24 H	643.5 ±274.7	2423.8 ±245.3	114.5 ±35.4	1.84±0.43	1.52±0.01
TMT 48 H	1009.4 ±190.8	2761.5 ±752.3	170.2 ±28.2	1.35±0.28	1.24±0.24
TMT168H	1022.3 ±532.9	2852.1 ±504.2	171.0 ±57.0	1.44±0.43	1.42±0.26
SAL IMM NO LD	1083.8 ±271.3	2281.1 ±340.9	178.5 ±30.8	1.35±0.52	1.12±0.51
SAL 24 H NO LD	1272.5 ±475.0	3406.9 ±1180.9	217.6 ±88.7	1.45±0.54	1.54±0.56
SAL 48 H NO LD	816.5 ±501.3	1857.6 ±919.1	141.5 ±42.8	1.46±0.69	1.27±0.65
SAL 168H NO LD	1215.4 ±552.7	2265.5 ±791.4	170.7 ±56.5	1.39±0.46	1.81±0.75
BA IMM NO LD	1125.5 ±349.0	2686.6 ±453.5	170.6 ±32.4	2.45±0.41	2.31±0.34
BA 24 H NO LD	565.8 ±129.5	2006.2 ±728.6	113.2 ±13.4	1.14±0.31	1.29±0.34
BA 48 H NO LD	441.9 ±273.9	1681.4 ±519.9	87.3 ±9.9	1.01±0.35	1.28±0.44
BA 168 H NO LD	1147.9 ±475.1	1877.2 ±831.8	144.8 ±47.4	1.89±0.12	1.64±0.16
TMTIMM NO LD	641.7 ±163.9	1562.0 ±589.5	105.6 ±23.8	1.28±0.07	1.51±0.06
TMT 24 H NO LD	408.4 ±214.5	771.0 ±318.3	78.5 ±27.4	1.62±0.01	2.08±1.0
TMT 48 H NO LD	359.1 ±171.7	580.7 ±227.6	48.2 ±11.0	0.34±0.001	0.49±0.15
TMT168H NO LD	734.8 ±347.4	2465.8 ±1033.8	123.9 ±51.6	1.86±0.3	1.88±0.4

TABLE 4.4: Quantitative Analyses of ENK immunoreactivity from the Basolateral (BLA), Central (CEA) and Medial (MEA) amygdaloid nuclei and nucleus accumbens core and shell (DO= optical density DI=integrated density SAL=Saline BA=Butyric Acid TMT=Predator odor NO LD= No light-dark box test).

dark box anxiety $F(1,18) = 5.018$, $p<.05$. Anxious mice displayed decreased ENK integrated density compared to mice termed non-anxious in the LD condition (See Figure 4.20).

Insert Figure 4.20 about here

Medial Nucleus of the Amygdala

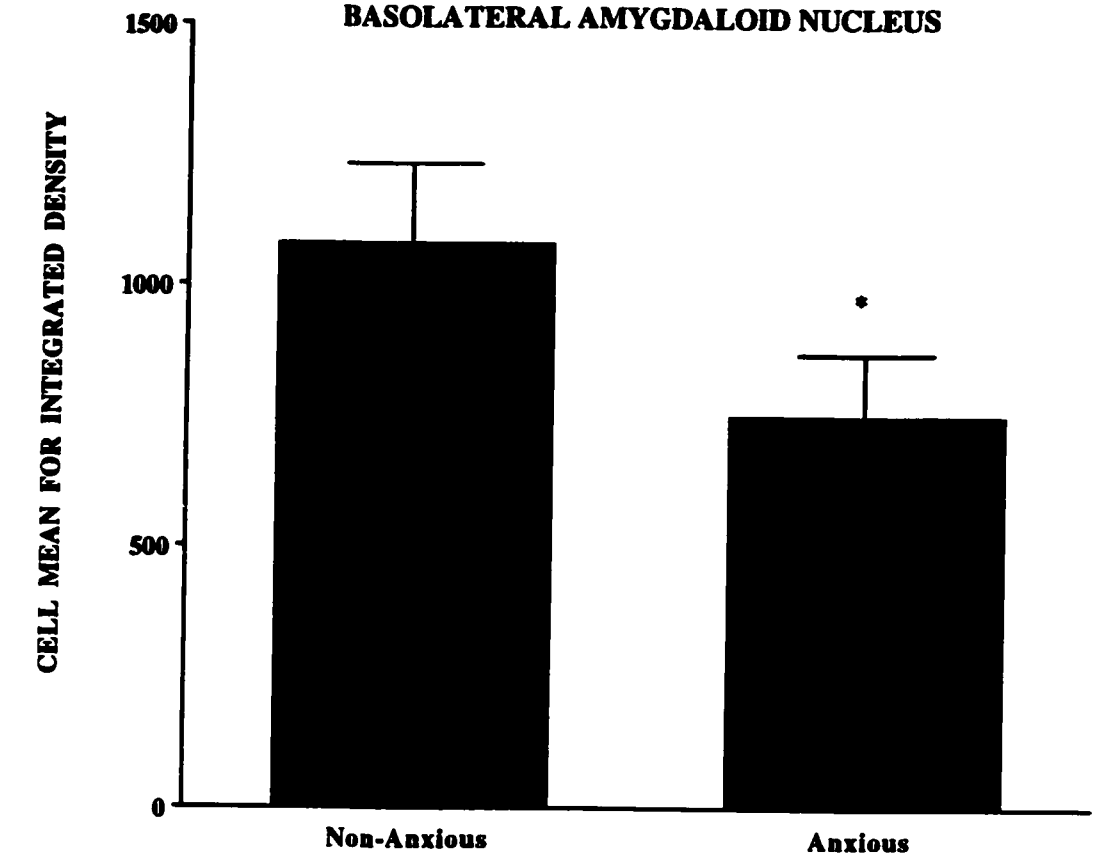
Analysis of variance of the integrated density of ENK mRNA from the MEA revealed a main effect of odor $F(2, 127) = 3.308$, $p<.05$. Mice exposed to TMT displayed decreased ENK gene expression compared to saline or butyric acid treated mice (See Table 4.4).

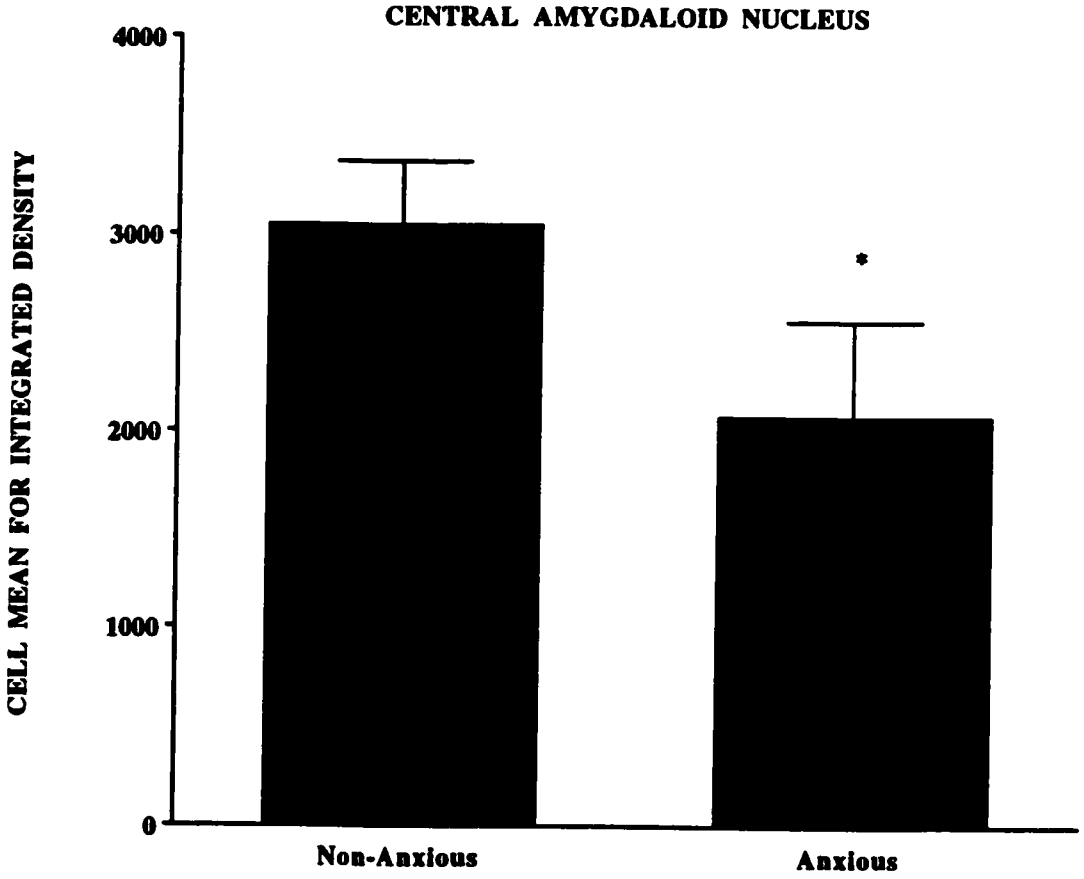
Central Nucleus of the Amygdala

Analysis of variance of the integrated density of ENK mRNA from the CEA revealed a main effect of light-dark box exposure $F(1, 102) = 6.180$, $p<.01$. Mice tested in the light dark box displayed increased ENK compared to mice in the No LD control condition (See Table 4.4).

Analysis of variance of the integrated density of ENK in the CEA of mice in the No LD condition termed responders or non-responders to predator odor failed to reveal any differences among treatment conditions (data not shown). Analysis of variance of the integrated density of ENK in the CEA of mice in the LD condition termed anxious or non-anxious/responders or non-responders to predator odor revealed a main effect of light-dark box anxiety $F(1,17) = 5.509$. Mice termed anxious displayed decreased ENK compared to mice termed non-anxious in the LD condition (See Figure 4.20).

Figure 4.20: Cell mean (± S.E.M.) of ENK integrated density (μCi/gr * sq. pixel 10^3) in the basolateral (BLA) (top panel) and the central amygdaloid nucleus (CEA) (bottom panel) in mice classified as anxious or non-anxious in the light dark box following exposure to fox odor. Note: * depicts a statistically significant difference ($p < .05$) of light dark box anxiety following TMT exposure on ENK mRNA expression in the amygdaloid complex.





LIGHT-DARK BOX BEHAVIOR

Acoustic Startle

The startle apparatus was previously described in Experiment 2. Mice were first habituated to the glass petri dish placed in their home cage with no odors present for 2 minutes (n=27, 15 mature mice (6 months old) and 12 juvenile mice (3 months old)) or 10 minutes (n=32, 17 mature mice (6 months old) and 15 juvenile mice (3 months old)) on three consecutive days. On each habituation trial mice were transported to another testing area where baseline startle measures were assessed. Following habituation procedures, mice were randomly assigned to predator, butyric acid, or saline treatment conditions. Mice were then transported immediately (5 minutes) to the second experimental room where acoustic startle was assessed Immediate (5 minutes), 24 hours, 48 hours and 168 hours following odor presentation. All animals were then sacrificed using CO_2.

Data Analysis

Startle scores were converted to a percentage of baseline and subjected to separate ANOVAs as juvenile 2 minute or 10 minute exposure and mature 2 minute or 10 minute exposure with repeated measures over days (4). Fisher's least significant difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

RESULTS

Analyses of variance of baseline startle amplitudes associated with 2 or 10 minute odor exposure in juvenile or mature mice failed to reveal any significant differences among assigned treatment groups ($F<1$, data not shown). However, analysis of variance of the baseline ASR profiles prior to experimental treatment did reveal, however, a significant main

effect of age, $F(1,54) = 27.502$, $p<000$, whereby juvenile mice had significantly greater mean ASR scores compared to mature mice.

Analyses of variance of the startle amplitude associated with 2 minute or 10 minute odor exposure in juvenile mice failed to reveal any differences among treatment conditions over all 4 test sessions. Analysis of variance of startle amplitudes of juvenile mice exposed to a 2 minute odor session on the immediate day revealed a main effect of odor $F(2, 11) = 6.681$, $p < .01$. Fisher's LSD multiple comparisons revealed an increase in startle amplitude in mice exposed to 2 minutes of predator odor relative to saline or butyric acid treated mice during the immediate test session (see Figure 5.1). Fisher's LSD multiple comparisons revealed a decrease in startle amplitude in mice exposed to 10 minutes of predator odor relative to saline or butyric acid treated mice during the immediate test session only (See Figure 5.2).

Insert Figures 5.1 and 5.2 about here

Analyses of variance of the startle amplitude associated with 2 minute odor exposure in mature mice revealed a main effect of odor $F(2, 8) = 4.769$, $p < .05$. Fisher's LSD multiple comparisons revealed an increase in startle amplitude in mice exposed to 2 minutes of predator odor relative to saline or butyric acid treated mice immediately, 24 and 48 hours following odor presentation. Mice exposed to 2 minutes of butyric acid demonstrated an increase in startle amplitude relative to saline treated mice 48 hours following odor presentation (See Figure 5.3).

Insert Figure 5.3 about here

Figure 5.1: Mean (± S.E.M.) startle amplitude (% baseline startle) among juvenile CD-1 mice following a 2 minute exposure to saline (SA), butyric acid (BA) or fox odor (TMT) and assessed for startle reactivity immediately, 24 hours, 48 hours and 168 hours following odor exposure. Note: * depicts a statistically significant difference ($p < .05$) in the acoustic startle response (ASR) among mice exposed to TMT relative to saline or butyric acid treatment.

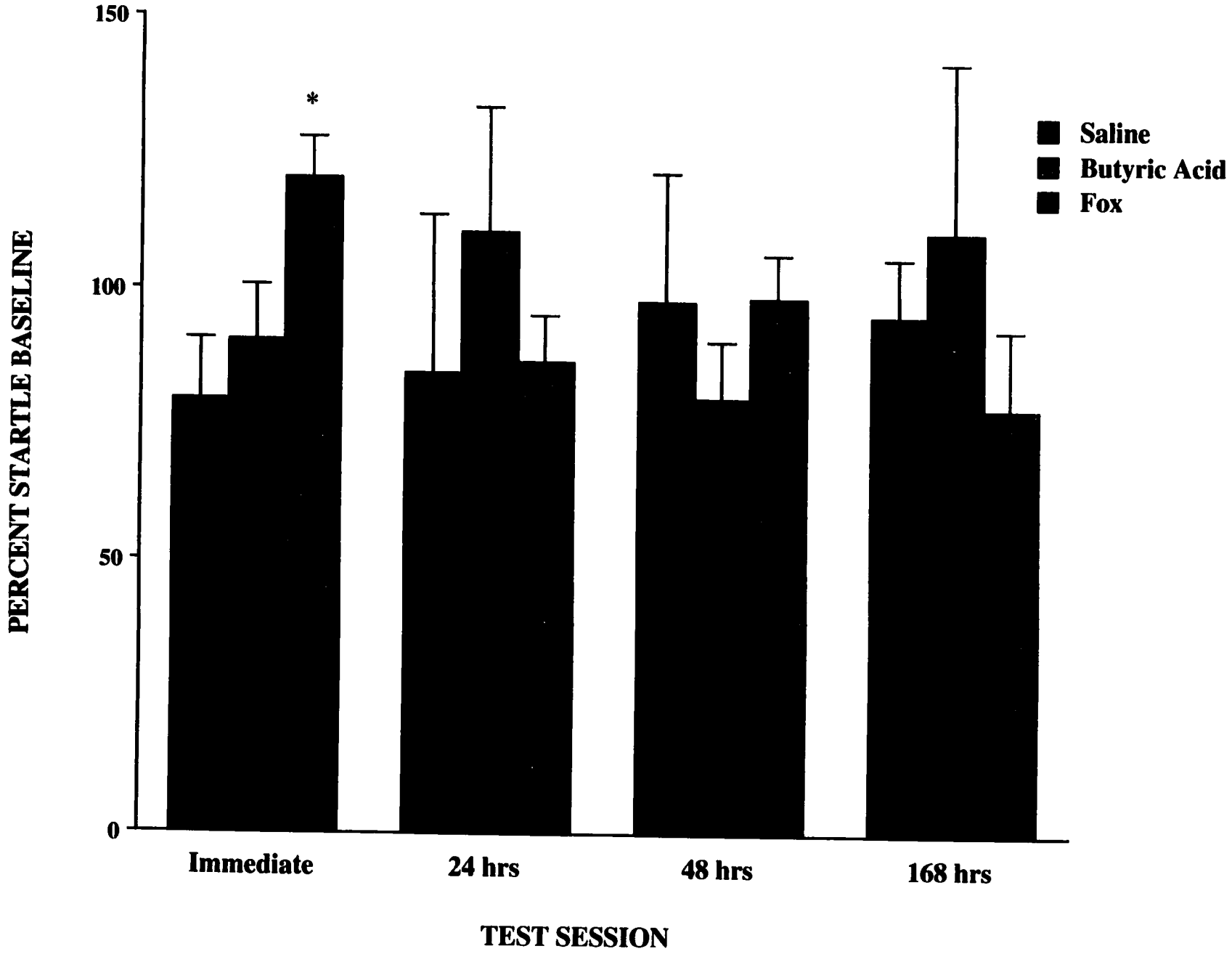

150
100
50
0
PERCENT STARTLE BASELINE
*
Saline
Butyric Acid
Fox
Immediate
24 hrs
48 hrs
168 hrs
TEST SESSION

Figure 5.2: Mean (± S.E.M.) startle amplitude (% baseline startle) among juvenile CD-1 mice following a 10 minute exposure to saline (SA), butyric acid (BA) or fox odor (TMT) and assessed for startle reactivity immediately, 24 hours, 48 hours and 168 hours following odor exposure. Note: * depicts a statistically significant difference ($p < .05$) in the acoustic startle response (ASR) among mice exposed to TMT relative to saline or butyric acid treatment.

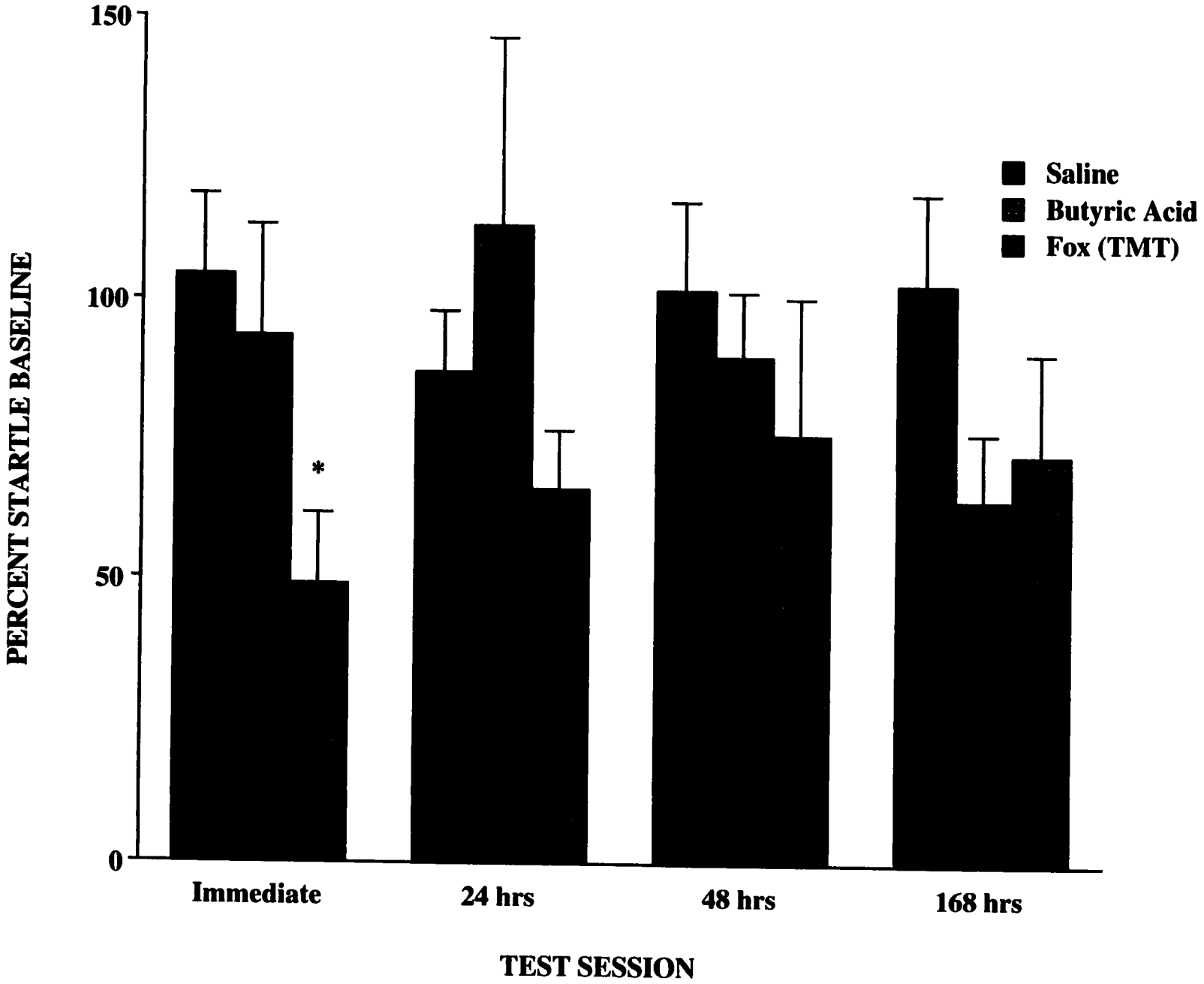
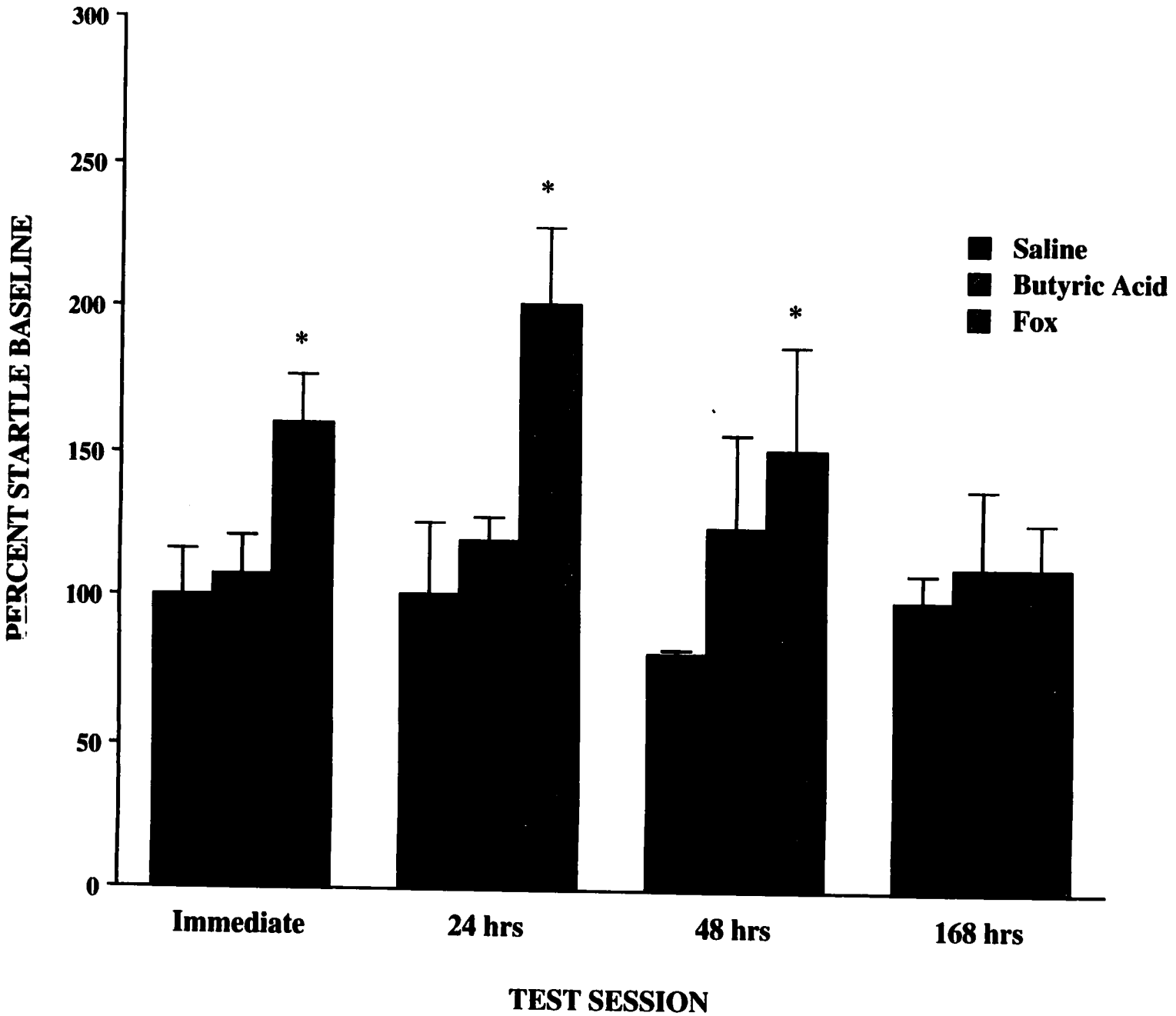

150
100
50
0
PERCENT STARTLE BASELINE
*
Saline
Butyric Acid
Fox (TMT)
Immediate
24 hrs
48 hrs
168 hrs
TEST SESSION

Figure 5.3: Mean (± S.E.M.) startle amplitude (% baseline startle) among mature CD-1 mice following a 2 minute exposure to saline (SA), butyric acid (BA) or fox odor (TMT) and assessed for startle reactivity immediately, 24 hours, 48 hours and 168 hours following odor exposure. Note: * depicts a statistically significant difference ($p < .05$) in the acoustic startle response (ASR) among mice exposed to TMT relative to saline treatment.


300
250
200
150
100
50
0
PERCENT STARTLE BASELINE
*
*
*
Saline
Butyric Acid
Fox
Immediate
24 hrs
48 hrs
168 hrs
TEST SESSION

Analyses of variance of the startle amplitude associated with 10 minute odor exposure in mature mice revealed a main effect of odor $F(2, 14) = 6.970$, $p < .01$. Fisher's LSD multiple comparisons revealed an increase in startle amplitude in mice exposed to 10 minutes of predator odor relative to saline or butyric acid treated mice immediately, 48 and 168 hours following odor presentation. Mice exposed to 10 minutes of butyric acid demonstrated an increase in startle amplitude relative to saline treated mice 168 hours following odor presentation (See Figure 5.4).

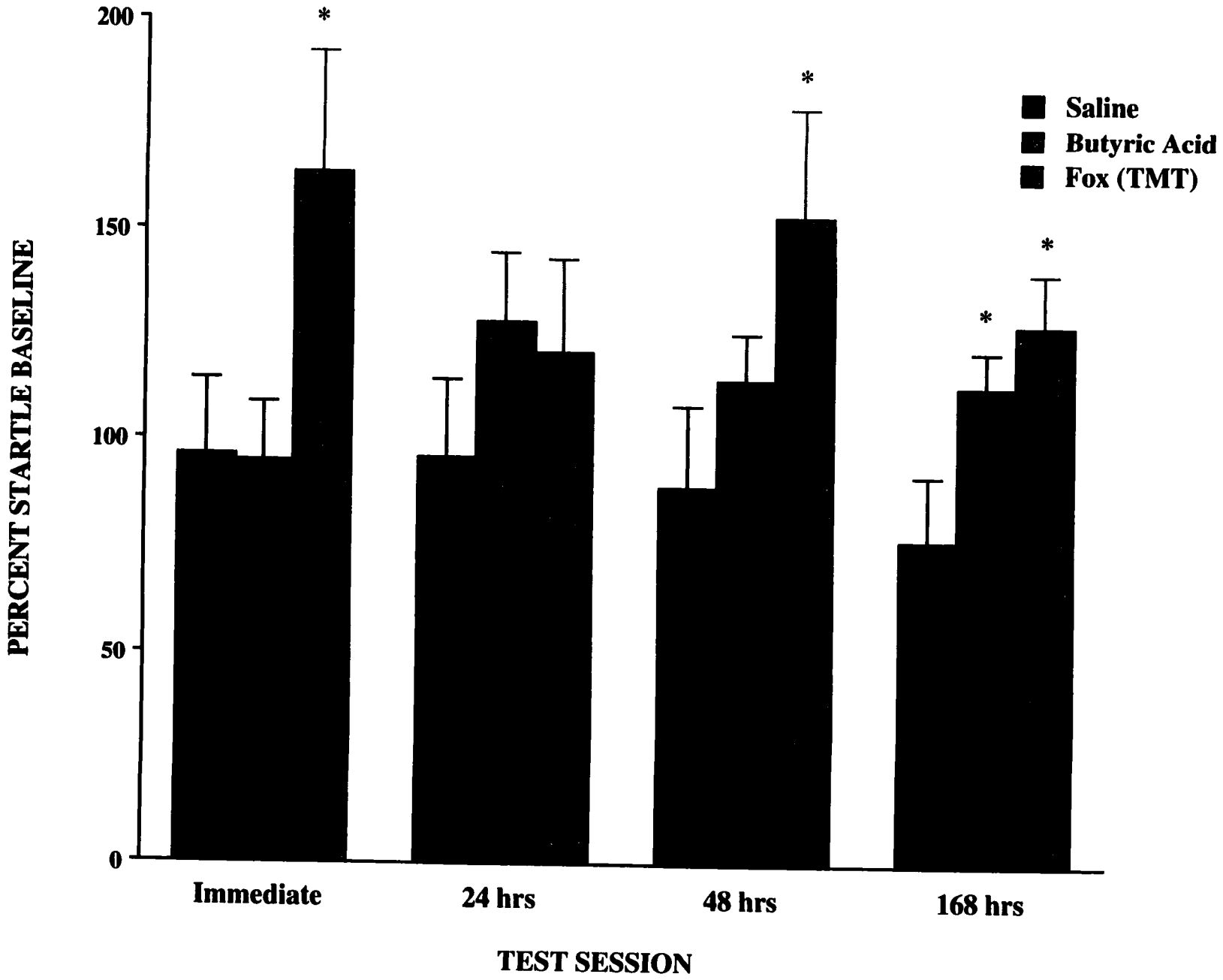
Insert Figure 5.4 about here

Intracranial Self-Stimulation (ICSS)

Surgery

Eighteen CD-1 mice were anesthetized with Halothane, B.P. (2-bromo-2-chloro-1, 1,1-Trifluoroethane) and stereotaxically (David Kopf Micromanipulator fitted with a Stoelting gas adapter for mouse ventilation) implanted with a bipolar stainless steel electrode (0.5 mm tip to tip, Plastic One, Roanoke, Va.) in the dorsal aspects of the mesencephalon. Stereotaxic coordinates for the dorsal A10 area were A.P. -3.0 to -3.2 mm from Bregma, L. +0.5 mm from the midline and V. -4.8 mm from flat skull surface. Mice were maintained on warm heating pads for three days postoperatively and supplemented with a wet mash diet (Meretene) prior to being returned to the main animal housing area. Behavioural tests commenced following a 10-day recovery period. All mice were individually housed postoperatively.

Figure 5.4: Mean (± S.E.M.) startle amplitude (% baseline startle) among mature CD-1 mice following a 10 minute exposure to saline (SA), butyric acid (BA) or fox odor (TMT) and assessed for startle reactivity immediately, 24 hours, 48 hours and 168 hours following odor exposure. Note: * depicts a statistically significant difference ($p < .05$) in the acoustic startle response (ASR) among mice exposed to butyric acid or TMT relative to saline treatment.


200
150
100
50
0
PERCENT STARTLE BASELINE
*
*
*
*
Saline
Butyric Acid
Fox (TMT)
Immediate
24 hrs
48 hrs
168 hrs
TEST SESSION

Self-Stimulation Training

The self-stimulation chambers consisted of 30 cm (diameter) x 24 cm (high) white polyethylene tubs. The floor of each tub contained a 2 cm well positioned in the center of the chamber. Head dipping through a distance of 1 cm interrupted a photobeam, resulting in the delivery of electrical brain stimulation through a mercury swivel commutator connected to a constant current stimulator (Schnabel Electronics, Saskatoon, Saskatchewan). Brain stimulation consisted of a monophasic square wave with a pulse width of 0.3 msec, a current intensity of 70 μA and 0.1 sec train duration. All mice were initially trained to respond at a frequency of 80 Hz for 15 minutes, until stable responding was elicited (<10% variation on three successive days). Preliminary studies revealed that frequencies exceeding 80 Hz (e.g., 90 Hz and 100 Hz) disrupted responding for brain stimulation from the VTA (reduced head-dipping) and provided behavioural indices of excessive activation. Such performance alterations at the upper end of the stimulation frequency distribution appeared to be associated with a departure from effective stimulation parameters previously documented in this laboratory (Zacharko et al., 1990). The adoption of the 20 Hz frequency represented the minimal stimulation parameter effective in provoking moderate rates of responding. Following self-stimulation training, baseline descending and ascending frequency response functions were determined for each animal. In particular, the stimulation frequency was set at 80 Hz (70 μA) and decreased in 10 Hz steps at 3-minute intervals over 7 trial blocks. Responding for brain stimulation was recorded at each 3 minute interval at a frequency range between 80 Hz and 20 Hz. Following completion of the descending phase of the test session, brain stimulation frequencies were increased by 10 Hz steps from 20 Hz to 80 Hz and

responding was again determined at 3 minute intervals. Self-stimulation performance was averaged across the descending and ascending frequency modes and a response curve was established for each animal according to previously established protocols (Fibiger et al., 1981). The scores of all animals were subsequently averaged for the respective treatment conditions and electrode placements. The selection of 3-minute trial blocks for each of the stimulation frequencies presented to CD-1 mice was predicated on previous determinations in this (Bowers et al., 1987) and other laboratories (Nakajima & O'Regan, 1991).

Following determination of baseline self-stimulation performance, mice were habituated to the glass petri dish placed in their home cage with no odors present for 10 minutes on three consecutive days. On each habituation trial mice were transported to another testing area where self-stimulation performance was again assessed to ensure animals maintained previously stable levels of responding. Following habituation procedures, mice were randomly assigned to saline (n=6), butyric acid (n=6) or predator (n=6), treatment conditions. Following odor exposure, mice were assessed for self-stimulation performance. Mice were subsequently reassessed 24 hours, 48 hours and 168 hours following initial odor exposure. Following behavioural testing, animals were perfused intracardially with physiological saline and 10% formalin. Brains were excised from the cranial cavity and placed in formalin for at least 2 weeks. The brains were subsequently blocked, frozen on a microtome and coronal sections (40 μm) were mounted and stained with cresyl violet to verify electrode placement.

Data Analysis

All ICSS scores were converted to a percentage of maximum Baseline (80 Hz) and subjected to analysis of variance (ANOVA) with repeated measures over frequencies (7) and test sessions (4). Fisher's least significant difference (LSD) multiple comparisons were employed where appropriate and the 0.05 level of significance was adopted for all comparisons.

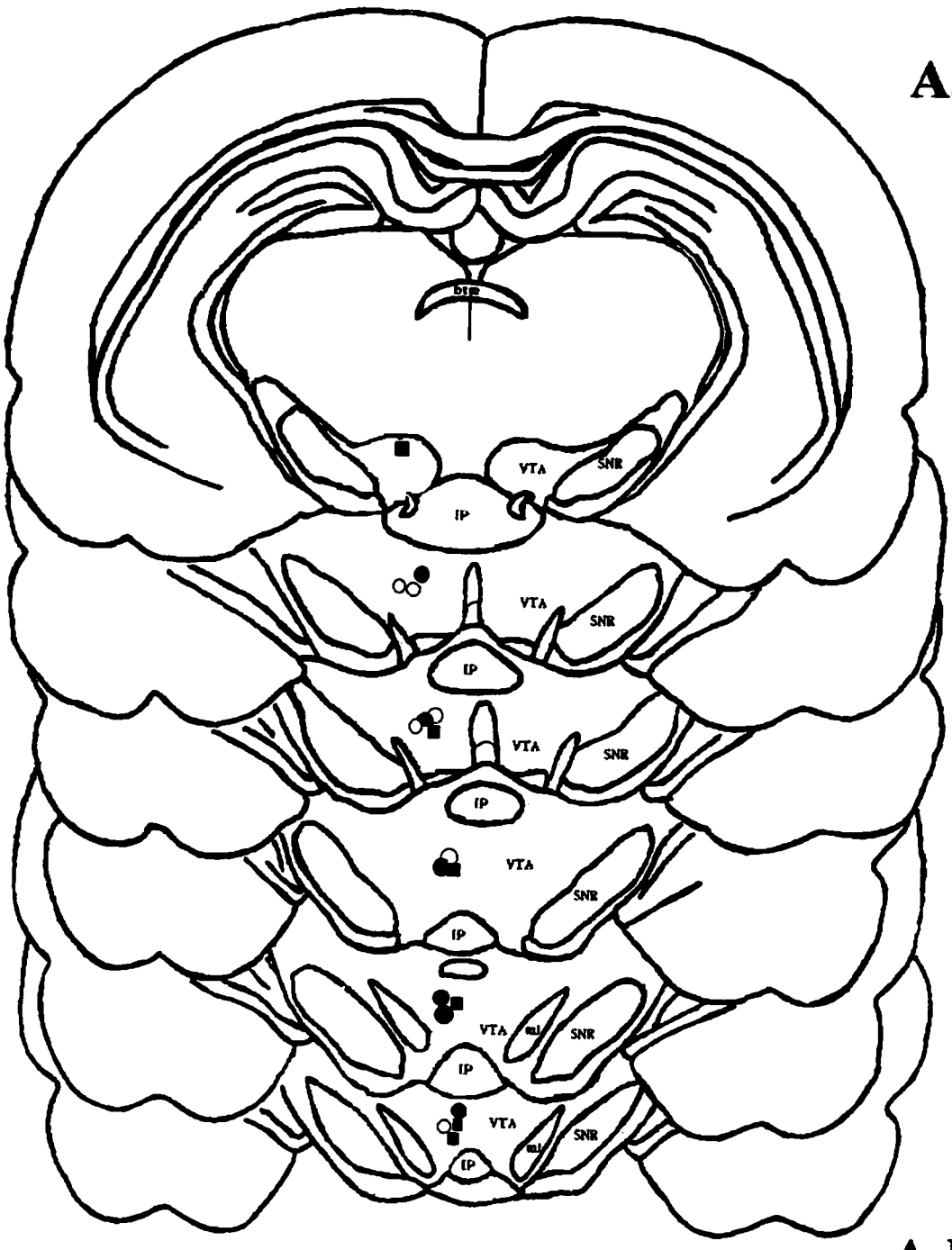
RESULTS

Histological analyses verified electrode placement in the dorsal aspects of the VTA. Six animals were not included in the statistical analyses due to electrode placements in nuclei other than the dorsal aspects of the VTA. Schematic representations of the distribution of dorsal A10 electrode positions are presented in Figure 6.1.

Insert Figure 6.1 about here

Analysis of variance revealed that baseline frequency response curves among mice responding for brain stimulation from the dorsal aspects of the VTA in the saline, butyric acid or predator treated conditions did not differ from one another, $F(2, 15)= 0.564$, $p>.05$. As expected, self-stimulation performance from the dorsal aspects of the VTA varied with stimulation Frequency $F(6, 90)= 68.607$, $p<.0001$ such that increments in self-stimulation performance were detected as self-stimulation frequency increased. The performance of mice responding for brain stimulation from the dorsal aspects of the VTA were expressed as a percentage of maximal baseline performance achieved at 80 Hz (See Figure 6.2).

Figure 6.1: Schematic representation (rostral-caudal from Bregma) depicting electrode placements in the dorsal VTA of mice exposed to 10 minutes of saline (SA), butyric acid (BA) or fox (TMT) odor. Open circles denote SA treated mice, closed circles represent mice exposed to BA and closed squares represent mice exposed to TMT. Brain areas identified include the ventral tegmental area (VTA), substantia nigra (SN) and the interpeduncular nucleus (IP).


A.P. -3.0
VTA
SNR
IP
VTA
SNR
IP
VTA
SNR
IP
VTA
SNR
IP
VTA
SNR
IP
VTA
SNR
IP
A.P. -3.8

Insert Figure 6.2 about here

Analysis of variance of self-stimulation scores revealed a significant main effect of Day $F(3, 270)= 5.111$, $p<.01$, Frequency $F(6, 270)= 36.157$, $p<.001$, a Frequency x Day interaction $F(18, 270)= 1.842$, $p<.05$, and an Odor x Frequency interaction $F(12, 270)= 3.50$, $p<.001$. The performance of mice responding for brain stimulation from the dorsal aspects of the VTA did not differ following butyric acid or TMT exposure. Both butyric acid and TMT exposure reduced self-stimulation performance at 80 Hz only relative to saline treated mice immediately, 24 and 48 hour post-odor applications. No significant differences among treatment groups were observed at the 168 hour test interval (See Figure 6.3).

Insert Figure 6.3 about here

DISCUSSION

The data of Experiment 4 revealed that exposure of CD-1 mice to the novel odor, butyric acid, or the predator odor, TMT, elevated risk assessment and suppressed non-defensive behaviours relative to neutral odor application. Mice exposed to butyric acid or the synthetic predator odor, TMT, contacted the odorant cloth less, displayed increased frequency of stretch attends, decreased rearing behaviour and displayed enhanced defensive burying relative to mice exposed to saline. These data are consistent with previous investigations examining the behavioural profiles of mice in response to cat odor. In these studies, while mice display increased risk assessment behaviours to predator odor they also exhibit similar

Figure 6.2: Baseline self-stimulation performance (± S.E.M.) of mice responding for brain stimulation from the dorsal aspects of the VTA in a descending and ascending frequency response curve. Animals were permitted to respond for brain stimulation for 3 minutes at each of the frequency parameters employed. Self-stimulation performance is expressed as a percentage of maximal baseline at 80 Hz. Note: saline, butyric acid or predator odor designations provided for the mice implanted with a stimulating electrode in the dorsal VTA region reflect group assignment prior to odor exposure.

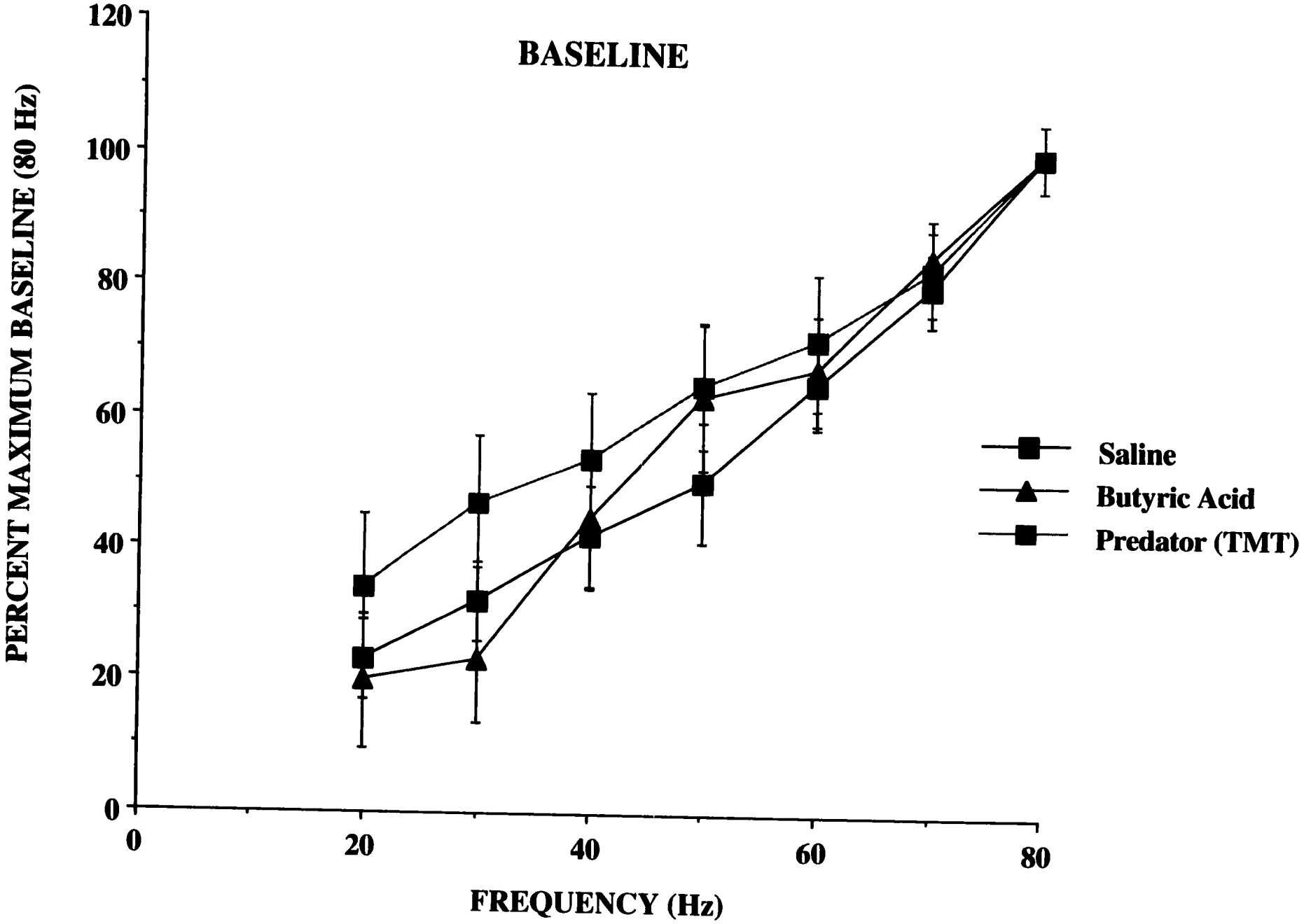
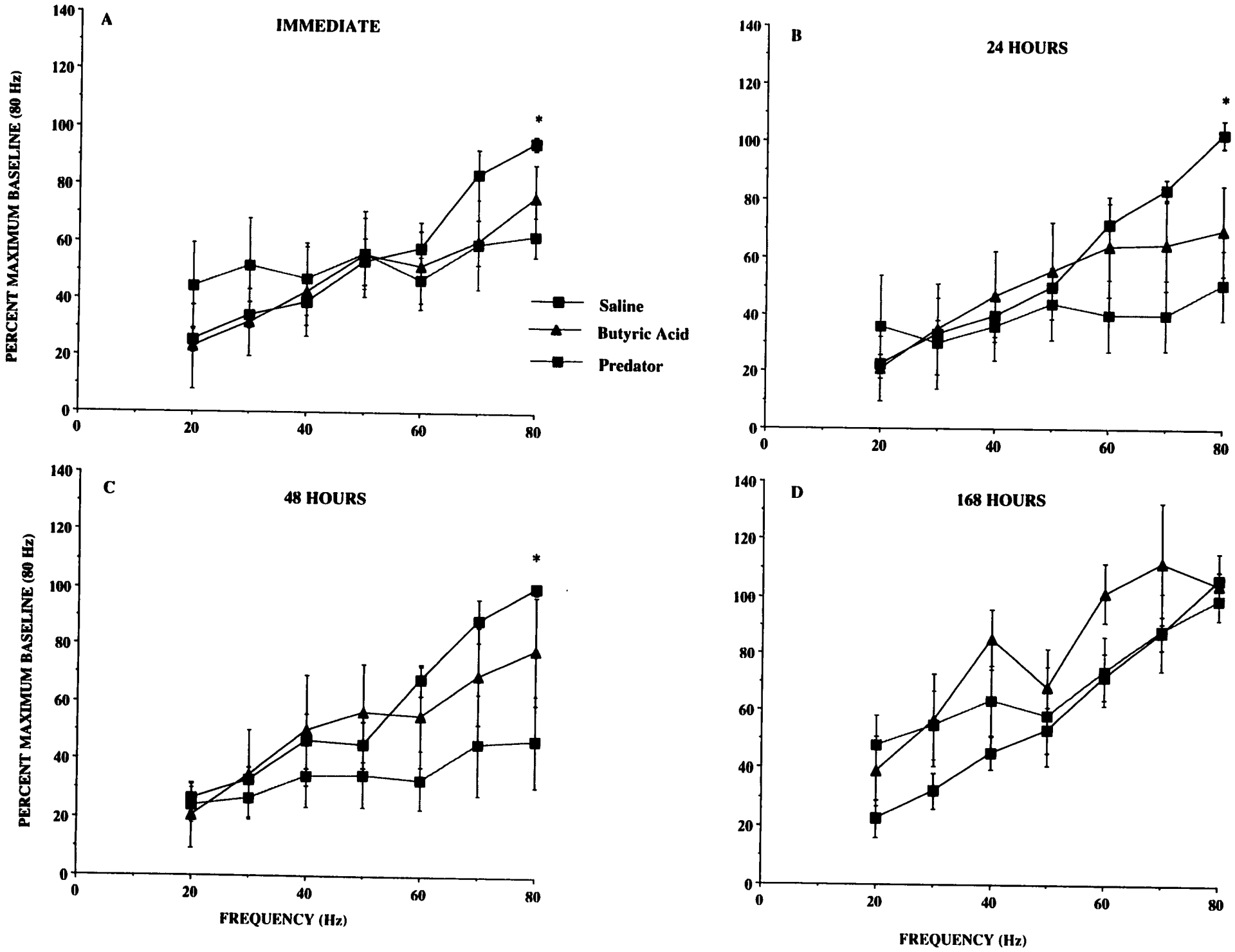

BASELINE
PERCENT MAXIMUM BASELINE (80 Hz)
120
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
Saline
Butyric Acid
Predator (TMT)

Figure 6.3: Self-stimulation performance (± S.E.M.) of mice responding for brain stimulation from the dorsal A10 area expressed as a percentage of maximal responding of baseline at 80 Hz immediately (5 minutes), 24 hours, 48 hours and 168 hours following a 10 minute exposure to saline, butyric acid or predator odor. Animals were permitted to respond for brain stimulation for 3 minutes at each of the frequency parameters employed in a descending and ascending fashion. Note: * depicts a statistically significant decrease ($p < .05$) in responding for brain stimulation at 80 Hz among mice exposed to TMT or butyric acid relative to saline treatment.


A
IMMEDIATE
B
24 HOURS
C
48 HOURS
D
168 HOURS
PERCENT MAXIMUM BASELINE (80 Hz)
FREQUENCY (Hz)
Saline
Butyric Acid
Predator
*
0
20
40
60
80
100
120
140

reactions to a variety of other novel odors (Blanchard et al., 1990; Kemble, 1994; Kemble & Bolwahnn, 1997). These behavioural deficits were exaggerated in mice exposed to TMT relative to mice exposed to butyric acid. For example, TMT depressed the frequency and duration of cloth contact and elevated the frequency and duration of freezing and defensive burying among CD-1 mice, more effectively than butyric acid. The stretched attend posture, freezing, as well as defensive burying, can be observed in rats in response to electric prods, novel and predator odors and such behaviors are attenuated with benzodiazepines (Blanchard et al., 1990; Molewijk et al., 1995). It should be underscored that defensive burying of electric prods was enhanced with increased intensity of electric shock (Blanchard et al., 1990). To all intents and purposes, the presentation of butyric acid and TMT may induce a fear gradient that underlies the appearance and relative intensity of defense patterns of CD-1 mice in response to odor exposure.

Exposure of CD-1 mice to either butyric acid or TMT induced anxiety in the light-dark paradigm during the immediate test interval only. These data parallel previous findings of Zangrossi and File (1992) who reported that exposure of rats to cat odor induced anxiety in the social interaction and elevated plus maze paradigms 1 hour but not 24 hours following odor exposure. In the present investigation, mice exposed to either butyric acid or TMT took longer to reenter the lit compartment and spent less cumulative time in the light section of the apparatus relevant to mice exposed to saline. The similar effects of butyric acid and TMT on cumulative time spent in light follows from the previous observation that time in light, is more sensitive to mild stressor imposition relative to transition scores (Kilfoil et al., 1989; MacNeil et al., 1997). The mere presentation of novel odors is sufficient to induce anxiety in the light-dark paradigm. In contrast, exposure of mice to TMT induced an anxiogenic profile

paralleled in both transition scores as well as cumulative time in light. For example, in CD-1 mice, TMT exposure was associated with decreased light and dark compartment transitions relevant to butyric acid or saline applications. It has been posited that a decrease in transition frequency is an index of reduced exploration (Crawley & Goodwin, 1980). Indeed, exposure of rats and mice to predators (Adamec, 2001) and predator odors (Berton et al., 1998) decreased exploration in novel environments. In experiment 4, CD-1 mice exposed to TMT also spent less time in the lit portion of the apparatus relevant to mice exposed to butyric acid. This exaggerated anxiogenic influence of TMT was delayed and did not emerge until the second concurrent 5 minute test session. The appearance of enhanced anxiety or the lack of habituation of behavioural responses to TMT exposure is supported by the general finding that responses to novel odors but not predator related cues habituate over time (Vernet-Maury et al., 1992; Zangrossi & File, 1992).

Among CD-1 mice exposed to TMT, two general patterns of defensive behaviour were observed. While all mice attempted to bury the odorant cloth, individual differences were observed as to the frequency and duration of defensive burying. Moreover, not all mice exhibited freezing behaviour, following attempts to conceal the odorant cloth. Mice were classified as responders or non-responders to TMT based on the duration of freezing. Non-responders froze for less than 1 minute, averaging approximately 30 seconds, while responders froze for more than 1 minute, averaging approximately 300 seconds, during the total 600 second odor exposure. The observation that some mice did not exhibit freezing behaviour parallel a previous study by Morrow et al. (2000) who employed the same amount of TMT (35 µl) purchased from the same distributor (i.e., PheroTech), in a similar manner

(i.e. filter paper), albeit in a novel environment (i.e., open field), to rats. In the study by Morrow et al. (2000), TMT did not induce alterations in freezing behaviour, grooming, vertical or horizontal activity of rats. Blanchard's group (2001) maintains that the defense patterns of laboratory mice and rats to highly threatening stimuli may vary across and within species. For example, mice display increased risk assessment and freezing behaviour, while rats, unlike mice display alarm vocalizations. Commensurate with these findings, responders spent less time in the light chamber of the light-dark box than mice displaying decreased freezing episodes during odor presentation. This effect was most pronounced during the immediate test session coinciding with increased anxiety observed in the light-dark paradigm. In contrast, Hogg and File (1994) classified rats as responders or non-responders to cat odor based upon the time sheltering during a 15 minute cat odor exposure. Both cat odor responders and non-responders displayed anxiety in the elevated plus maze. While both fear and anxiety promote similar symptoms, fear is regarded to be more stimuli specific than anxiety (Davis, 1992). To the contrary, Zangrossi & File (1994) reported responders demonstrated increased fear to a subsequent neutral odor following a 60 minute exposure to cat odor. Thus, the duration of psychogenic stressor experience, as well as, the initial response levels were prominent factors influencing behavioural sensitization and conditioned fear responses. To be sure, various procedural differences exist between these studies and our own, including the species used, the duration and nature of psychogenic stressor imposition and inter-paradigm differences in the assessment of anxiety. In effect, the demonstration of freezing behaviour in CD-1 mice, indicative of enhanced fear generated by predatory cues, influenced immediate behavioural reactivity or anxiety per se to subsequent novel environmental challenges.

In accordance with the aforementioned findings, examination of the pattern of FRA immunoreactivity from mesocorticolimbic sites in response to odor exposure and light dark box anxiety revealed distinct patterns of neuronal activation underlying the expression of behavioural change. The Fos family of transcription factors, including c-fos, Fos B, Fra-1 and Fra-2, act in concert with the Jun family to form the AP-1 heterodimer complex which binds to specific DNA promotor/enhancer regions of various genes, up-regulating or down-regulating their expression (see Herrera & Robertson, 1996 for review). These transcription factors are believed to induce transduction cascades coupling external stimuli to the long-term responses of neurons necessary for long-term memory and integration of fear and anxiety (Bruijnzeel et al., 1999; Herdegen & Leah, 1998). It has been suggested, based on immunoelectronmicroscopic analyses, that FRA-1 participates in synapse to nucleus communication modulating behavioural experience-dependent hippocampal long-term potentiation (Paratcha et al., 2000). Fos and fos related antigens are expressed rapidly and transiently in response to a variety of stimuli (Herdegen & Leah, 1998; Morgan & Curran, 1995), habituate with repeated exposure (Ryabinin et al., 1999) and the number of Fos expressing cells is dependent on the intensity of the experience (Campeau & Watson, 1997). The results that Fos is down-regulated following repeated exposure to the same stimuli (Ryabinin et al., 1999) are contrasted with observations that Fos is up-regulated following exposure to a conditioned stimulus (Campeau et al., 1991). Enhanced Fos positive neurons were noted in the mPFC, accumbens and amygdala to a shock prod among rats previously exposed to footshock 2 weeks earlier. These data suggest that altered reactivity to stressors were precluded by enhanced neuronal activation although in some cases behavioural sensitization was not expressed (Bruijnzeel et al., 1999). Indeed, in the present investigation,

exposure of CD-1 mice to TMT increased FRA from the infralimbic and prelimbic cortices as well as the VTA and mesolimbic shell of the nucleus accumbens relative to saline or butyric acid treated mice. This effect was most pronounced on the immediate day and no differences among treatment groups were evident 24 hours following odor exposure. Within the mPFC, light dark box exposure in mice was associated with enhanced FRA. Moreover, enhanced freezing was associated with elevated mPFC FRA immunoreactivity among mice exposed to fox odor. Anxious mice exhibited enhanced FRA in the shell of the accumbens relative to their non-anxious counterparts. It will be recalled that both butyric acid and TMT exposure were effective in inducing anxiety in the light-dark box relative to saline treated mice. Finally, a 10 minute TMT exposure was more effective in enhancing FRA from the mPFC, but not the VTA or core and shell of the accumbens, than a 5 minute exposure TMT session reaffirming the increased sensitivity of the mPFC to stressor applications (Deutch et al., 1991).

The results of Experiment 4 showed that exposure of mice to TMT results in transitory elevations of FRA in the mPFC and its efferent projection sites. The mPFC receives projections from VTA DA neurons and somatosensory cortex and in turn projects to the basolateral amygdala (BLA). The mPFC is an important neural interface between the somatosensory cortex and amygdala (Conde et al., 1995; Emson & Koob, 1978; McDonald et al., 1996) and mediates unconditioned inhibition of enhanced anxiety (Koch & Bubser, 1994). Interestingly, the pattern of FRA immunoreactivity in the amygdala was opposite to that of the mPFC and VTA. There was no effect of odor on FRA expression from the CEA or the BLA while light dark box exposure was associated with reduced FRA expression from these same nuclei. In a like vein, conditioned fear did not result in c-fos alterations in the basolateral or central amygdaloid nucleus in rats (Campeau et al., 1997; Rosen et al., 1998). These data

parallel previous research which report phasic activation of amygdala neurons (except medial amygdaloid nucleus) to novel or threatening stimuli, the function to of which is to preserve neuronal responsiveness in the event additional threats are encountered (Davis et al., 1998; Dielenberg et al., 2001). For example, 20 minutes of gentle handling increased DA availability in the nucleus accumbens and amygdala as revealed by HPLC and in vivo microdialysis in rats. Interestingly, such amygdaloid DA release was only evident during mild stressor application (Lanca et al., 1998) and can be contrasted with DA change within the nucleus accumbens which achieved a neurochemical asymptote after the cessation of the stressor (Enrico et al., 1998; Inglis & Moghaddam, 1999).

Among CD-1 mice exposed to TMT, odor exposure increased FRA expression in the core and the shell of the nucleus accumbens. Increased expression of FRA in the nucleus accumbens and mPFC may be a result of mesocorticolimbic DA activation which originates in the VTA (White, 1996). The shell of the nucleus accumbens is considered an extension of the amygdala, and is involved in the expression of motivational and reward changes. In contrast, the accumbal core and the striatum are involved in sensory motor integration (Barrot et al., 1999; Haber et al., 1995; Zahm et al., 2001). It should be noted parenthetically that novel odor-activated olfactory bulb cells exhibit increased Fos colocalized with tyrosine hydroxylase in DA neurons in awake rats (Guthrie & Gall, 1995). Indeed, it has been previously demonstrated that acute but not chronic TMT increased DA turnover in the mPFC and the BLA in rats (Morrow et al., 2000). Increased DA activation within the prefrontal cortex has also been demonstrated in rats exposed to fox urine (Hayley et al., 2001). However, TMT odor response as defined by enhanced freezing behaviour as well as anxiety in the light dark box was associated with increased FRA expression in the mesolimbic

shell and not the nigrostriatal accumbal core. Taken together, the severity of aversive stimulation appears to influence the nature of region specific neuronal activation.

It will be recalled that mesolimbic DA alterations and accompanying behavioural alterations in response to acute stressor encounters may be dependent on the intensity and duration of the stressor. For example, mild stressor experiences alter mesolimbic rather than nigrostriatal DA availability (Deutch et al., 1985, 1990, and 1991). Indeed, a 20 minute psychological stressor (e.g., exposure of rats to the auditory, visual and olfactory cues of conspecifics exposed to uncontrollable footshock) increased DA levels in the mesolimbic shell of the nucleus accumbens for 2 hours following stressor termination, while DA levels in the nigrostriatal core of the nucleus accumbens were unaffected as measured by in vivo microdialysis (Wu et al., 1999). Moreover, a modified 2-hour restraint session among rats permitting a margin of movement increased DA release in the nucleus accumbens while rats which were immobilized showed a decrease in DA release in the accumbens 40 minutes after exposure to the stressor (Kurata et al., 1993). Likewise, 15-30 minutes of restraint increased DA release within the mesoprefrontal cortex in rats while exposure to 60 minutes of the same stressor enhanced DA availability in the nucleus accumbens (Carlson et al., 1991). Likewise, alterations in DA activity within the mPFC, VTA and the nucleus accumbens have been associated with freezing (Pavlasevic et al., 1993; McIntyre et al., 1999) and increased startle in response to footshock and predator stress (Adamec et al., 1999; Borowski & Kokkinidis, 1996). Taken together, increased FRA expression in mesolimbic sites delineates the activation of neural circuits associated with fear and anxiety.

There is a considerable data base which characterizes release of mesolimbic DA in response to a variety of stressors and the potential influence of mesolimbic DA activity on

affect, motivation and anxiety (see Zacharko et al., 1995 for review). Neuropeptides modulate the activity of mesocorticolimbic DA activity. For example, enkephalin analogues administered into the VTA increase DA release and metabolism in the mPFC and nucleus accumbens (Kalivas et al., 1983; Dauge et al., 1992; Devine et al., 1993) while exposure to uncontrollable footshock also increases enkephalin release within this mesocortical site (Kalivas & Abhold, 1987). Conversely, systemic and intra-VTA administration of the opioid antagonists naloxone and naltrexone, attenuate stressor-evoked DA turnover in the mPFC and nucleus accumbens (Kalivas & Abhold, 1987). Increased enkephalin gene expression in central sites associated with fear and anxiety presumably underlies compensatory physiological responses that attenuate the deleterious effects of uncontrollable stressor applications (Dumont et al., 2000). In effect, neuropeptides colocalized with DA in mesolimbic sites may provide a neural correlate pertaining to the severity of an aversive encounter, influence the expression of behavioural change among infrahuman subjects and conceivably parallel symptom manifestation of clinical populations. For example, while life stressors provoke increased CSF β-endorphin levels among individuals with mild anxiety disorders (Baker et al., 1997; Darko et al., 1992; Eriksson et al., 1989; Goodwin et al., 1993; Westrin et al., 1999) panic patients and individuals with major depression display reduced CSF β-endorphin levels (Perez-Costillas et al., 1997; Zis et al., 1985).

In Experiment 4, ENK mRNA cell counts were increased in anxious mice in all sub-nuclei of the amygdala examined (CEA, BLA and MEA). This effect was most pronounced among mice in the immediate test interval. It should be emphasized that anxiety in the light dark box was only evident among mice during the immediate test session and no differences in

anxiety levels were noted among animals tested 24, 48 or 168 hours following odor experience. Moreover, examination of the double-labelling of FRA and ENK within the sub-areas of the amygdala revealed a similar pattern. Within the BLA and CEA, anxiety in the light-dark box was associated with increased ENK/FRA in mice previously exposed to TMT. Excessive fear levels, as indicated by enhanced freezing, was associated with reduced ENK mRNA expression from the CEA. These data parallel Cousino Klein et al. (1998) who reported that opioid blockade by peripheral naloxone administration enhanced unconditioned freezing in the home-cage following footshock imposition. However, conditioned freezing was associated with increased ENK mRNA from the CEA suggesting that ENK within this nucleus is associated with learning and memory (Petrovich et al., 2000). The effect of TMT exposure on the expression of ENK in the BLA is interesting as the lateral complex is considered to be an important interface between sensory brain areas and other amygdaloid nuclei including the CEA (Pitkanen et al., 1997) and is required for context and tone dependent conditioned freezing (Kim & Fanselow, 1992). It is well established that the CEA plays a prominent role in the acquisition and mediation of fear responses (Roozendaal et al., 1991). The CEA is the major output pathway to many subcortical nuclei that mediate fear related behaviours including freezing and startle (Rosen et al., 1998). The MEA has been implicated in a variety of behavioural responses including mating and aggression and induction of ENK in the MEA of anxious mice is consistent with a role of this nucleus in behavioural arousal and social memory (Kollack-Walker & Newman, 1995; Vochteloo & Koolas, 1987). The MEA is not specifically involved in fear as lesions confined to the MEA do not interfere with the expression or conditioning of fear responses (Oakes & Coover, 1997 c.f. Dayas et al., 1999).

Within the nucleus accumbens, only the nigrostriatal core hosted prominent ENK mRNA changes following odor exposure and behavioral testing. In this sub-region, ENK mRNA was elevated in mice exposed to the light-dark apparatus yet decreased in anxious mice, an effect most pronounced 24 hours following TMT exposure. These data suggest that the influence of odor and LD on ENK mRNA within the accumbal core is due to the motor enhancing properties of enkephalin within this nucleus (Johnson et al., 1995). Indeed, no effects were observed in the mesolimbic shell of the accumbens. However, exposure of mice to TMT increased FRA/ENK from the core and shell of the accumbens. This effect was most pronounced on the immediate test interval. Increased fear, as assessed by enhanced freezing, was effective in enhancing neuronal activation of ENK neurons in the shell but not the core of the accumbens. Taken together, the resultant pattern of neuronal activity within ENK cells parallels the induction of fear and anxiety within specific neural circuits activated by stressor imposition or more preferentially responsive to odors that acquired emotional significance. It should be underscored that the failure to differentiate basal ENK mRNA from neuronally activated ENK cells as with the quantitative analyses may provide inconsistencies in results between subjects. For example, no differences among treatment conditions were noted in ENK optical density from the nucleus accumbal core or shell. Within the amygdala, anxious mice exhibited decreased ENK in BLA and CEA. In effect, FRA may regulate stress responsive genes and mediate long-term changes in behaviour. To be sure, reexposure to mild stressor experiences would re-induce and exaggerate behavioural indices of anxiety accompanied by enhanced activation of ENK neurons.

Predator Exposure and Acoustic Startle

Experiment 5 demonstrated that exposure of mature CD-1 mice to 10 minutes of TMT increased acoustic startle 7 days post-stressor. To our knowledge this is the first study to demonstrate increased startle responsivity among CD-1 mice following fox odor presentation. Previous studies (i.e., Adamec et al, 1997) have demonstrated increased acoustic startle among rats previously exposed to a cat 1 week following predator exposure. The primary startle pathway includes only a few synaptic relays between cochlear nuclei, through a sensimotor interface (e.g., caudal pontine reticular nucleus) to muscles mediating the acoustic startle response (ASR) (Koch & Schnitzler, 1997; Walker et al., 1997). It has been reported that prior emotional learning can modify the ASR. Indeed, the association between the unconditioned stimulus (e.g. tone) and previous stressor experiences induces physiological changes in neurons of the basolateral amygdala (BLA) which enhance central amygdaloid (CEA) modulation of caudal pontine reticular neurons (PnC) (Davis et al., 1993). The ASR has been employed extensively to investigate classical conditioning (Campeau & Davis, 1995), drug-induced behavioural effects (Josselyn, et al., 1995), as well as habituation (Plappert et al., 1993), and sensitization (Sheets et al., 1998) in response to various neurogenic and psychogenic stimuli. The magnitude of the ASR in rats can be enhanced (i.e., sensitization) following exposure to footshock (Davis, 1990) and predator exposure (Adamec, et al., 1998). The ASR can likewise be attenuated (i.e., habituation) by the repeated presentation of auditory startle stimuli (e.g. white noise) (Plappert et al., 1993). The changes in magnitude of the ASR by administration of various drugs have been used to assess anxiety in infrahuman subjects. Drugs that increase anxiety, such as the panicogenic drugs yohimbine (Stine et al., 2001) and CCK-4 (Frankland et al., 1996; Frankland et al., 1997; Shlik et al.,

1999) also enhance the ASR in infrahuman and human subjects. In comparison, anxiolytic agents, including diazepam (Bitsios et al., 1999; Cannizzaro et al., 2001) and enkephalin μ and δ opioid agonists (Tilson et al., 1986; Vivian & Miczek, 1998) attenuate the ASR.

The data gleaned from experiment 5 replicate previous research demonstrating that exposure to psychogenic stressors produce behavioural alterations reminiscent of anxiety in mice and rats. In humans, conditioned fear or fear-enhanced startle has been linked to psychological disorders which eventuate in sustained and exaggerated reactivity to environmental stressors. For example, an enhanced startle response following a catastrophic life experience has been linked to post-traumatic stress disorder (Morgan et al., 1995) and panic (Grillon et al., 1994). In fact, a predisposition to react fearfully to stimuli or situations that are considered only mildly challenging to others has been proposed as a risk factor for anxiety disorders (Kagan et al., 1988; Whitehead et al., 1994). Indeed, adolescents with a parental history of anxiety disorders exhibited enhanced startle reactivity to a threat of an unpleasant air blast directed at the larynx (Grillon et al., 1998). Adult rats exposed to either a cat (Adamec et al., 1999) or a ferret (Plata–Salaman et al., 2000) exhibited enhanced startle reactivity in response to a subsequent auditory stimulus previously paired with the stressor. Taken together, the acoustic startle paradigm appears to be a reliable measure of anxiety among human and infrahuman subjects.

In the present investigation exposure of mature mice to 2 minutes of TMT increased startle immediately, 24 and 48 hours following odour presentation while a 10-minute exposure was effective in enhancing startle for 168 hours. Mature mice exposed to 2 minutes or 10 minutes of butyric acid demonstrated an increase in startle amplitude relative to saline

treated mice 48 and 168 hours following odor presentation, respectively. The delayed onset of enhanced ASR likely reflected a period of latency between stressor encounter and production of anxiety-like behaviour. It should be noted that startle stimuli themselves are aversive and may induce protracted states of fear or anxiety (Borszcz et al., 1989). These findings suggest that the duration and nature of stressor exposure and the association of the startle stimulus with prior psychogenic stressor application influences the temporal pattern of anxiety expression in CD-1 mice following stressor encounter.

The demonstration that exposure to a predator odour enhanced acoustic startle among mature but not juvenile CD-1 mice suggest that the influence of predatory cues on acoustic startle may be influenced by age-related factors. Indeed, Varty et al. (1998) demonstrated that acoustic startle was highest in adult rats (i.e., 11 months) although air puff reactivity was comparable among young (i.e., 3 months) and adult rats. Exposure of juvenile mice for 10 minutes to TMT decreased acoustic startle in the immediate test session, while this identical manipulation increased acoustic startle reactivity in mature mice for protracted intervals. It is unlikely that juvenile mice displayed enhanced habituation of the startle response relative to their mature counterparts. It has been demonstrated that repeated presentations of auditory stimuli induce habituation of the acoustic startle response (ASR) regardless of age (Pletnikov et al., 1996). However, short-term (within session) startle amplitude habituated (demonstrated by a decrease in startle) more quickly in aged rats (i.e., 26 months) relative to younger rats (i.e., 3 months) (Rinaldi and Thompson, 1983). While sensitization of the ASR in response to the presentation of a tone stimulus was more likely to occur in younger rats (Rinaldi and Thompson, 1983), it was shown that aged rats (i.e., 22 months) had 65% diminished basal startle reactivity compared to young rats (i.e., 3 months) (Varty et al.,

1998). Taking this data into perspective of the present experimental findings, it would be expected that mature mice would display reduced startle amplitudes to stressor imposition relative to juvenile mice. This was clearly not the case.

The decrease in startle amplitude in juvenile mice following a 10-minute exposure of TMT does not likely reflect a fleeting anxiolytic response of predator exposure. Indeed, examination of the behavioural repertoire of animals of similar age in experiment 4 revealed enhanced freezing in response to odour presentation followed by anxiety in the light-dark paradigm as indicated by reduced illuminated arena occupancy, light chamber re-entrance latency and reduced inter-compartmental transitions. The decrease in startle reactivity among juvenile mice suggests an increased sensitivity to stressor related cues and adoption of behavioural defence strategies, including enhanced freezing, incompatible with the elicitation of enhanced startle. Interestingly, Houston et al. (1999) demonstrated that aged rats exhibited less freezing behaviour than younger rats to a novel chamber previously paired with footshock. At elevated fear levels the amygdala enhances dorsolateral periaqueductal gray neuronal activity, which in turn acts in an inhibitory fashion to attenuate startle (Walker et al., 1997) and promote freezing (Graeff, 1994). Moreover, exposure of CD-1 mice to 2 minutes of predator exposure was not associated with freezing behaviour as immobility typically emerged following defensive burying, 4 to 5 minutes following stressor application. Interestingly, 2 minutes of TMT exposure was effective in enhancing startle reactivity among juvenile mice in the immediate post-stressor interval. Taken together, these findings suggest that the duration and severity of psychogenic stressor exposure and the association of the startle stimulus with prior TMT application age-dependently influences the temporal pattern of anxiety expression in CD-1 mice following stressor encounter.

Results from pharmacological studies suggest a role for mesolimbic DA activity in modulating the ASR in response to environmental manipulations. It has been demonstrated that VTA DA neuronal projections to the amygdala and mPFC regulate emotional reactivity to fear-inducing stimuli. (Borowski & Kokkinidis, 1996; Greba et al., 2001; Rosenkranz & Grace, 2001) The basolateral nucleus of the amygdala plays a significant role in affective behaviour that is thought to be regulated by dopamine (DA) afferents from the mPFC. It has been demonstrated that the mPFC attenuates BLA neuronal activity via recruitment of BLA interneurons that suppress cortical inputs (Rosenkranz & Grace, 2001). Indeed, acoustic startle was enhanced following a novel stressor (e.g., light), which was paralleled by increases in dopamine utilization in the prefrontal cortex (Anisman et al., 2000). It will be recalled that in rats, acute but not repeated exposure to TMT increased DA metabolism in the mPFC and the BLA compared to rats exposed to butyric acid (Morrow et al., 2000). Interestingly, preliminary data collected in this laboratory revealed that exposure of mature mice to 2 minutes of TMT on 3 consecutive days failed to enhance startle reactivity relative to repeated butyric acid or saline treatment immediately, 24 hours, 48 hours or 168 hours following termination of repeated odor presentations. In other investigations, systemic administration of SKF 82958, a D1 receptor agonist, significantly enhanced startle in rats while pre-treatment with SCH 23390, a DA receptor antagonist, lead to the blockade of enhancement by SKF 82958 (Meloni and Davis, 1999). Finally, microinjection of SCH23390 into the rat prefrontal cortex decreased prepulse inhibition of the ASR (Zavitsanou et al., 1999). Taken together, it should be considered that neuropeptides colocalized with dopamine, including enkephalin, modulate startle reactivity by modulating central DA activity.

Interestingly, there are data which outline age associated variations in DA release following stressor encounter as well as the influence of stressor parameters on DA function. For example, seventy-day old rats demonstrated significant footshock-induced increases in brainstem DA levels while one-year old rats showed no appreciable change (Welsh & Gold, 1984). Moreover, DA concentrations in the amygdala and striatum were significantly decreased in 24 month-old rats relative to 12 month-old rats (Miguez et al., 1999; Carfagna et al., 1985). It has been posited that reduced DA concentrations leave the organism less able to cope with stressor imposition and ultimately increase behavioural pathology (Zacharko & Anisman, 1991). It follows that the more persistent the amine depletion, the increased probability of psychopathology appearing. Indeed, the severity of the stressor experience may be quantitatively defined. Taken together, protracted indices of startle reactivity in response to aversive stimuli among infrahuman subjects appears to be dependent upon a neurochemical interaction between the PnC and the BLA/CEA, in addition to relay nuclei between the CEA and the PnC, such as the periaqueductal gray. The magnitude of the ASR in rats and mice can be modulated by age-dependent mesolimbic DA responsivity and precipitated by perceived stressor severity as defined by the intensity and duration of the initial stressor experience.

Predator Exposure and VTA ICSS

The data of experiment 6 revealed that predator odor and the novel odor butyric acid were ineffective in elevating self-stimulation reward thresholds from the dorsal VTA. However, other hedonic measures including sucrose consumption are influenced by predator exposure. For example, chronic but not acute exposure of male CD-1 mice to rat odor reduced sucrose intake (Calvo-Torrent et al., 1999). It should be considered that there are differences

between sucrose consumption and ICSS as hedonic processes. Indeed, exposure of rats to chronic mild stressors, including periods of food and/or water deprivation soiled cage, light/dark reversal, and confinement to small cages was effective in reducing the intake of sucrose solution but ineffective in disrupting hypothalamic ICSS in rats (Nielson et al., 2000). Unfortunately, the influence of psychogenic stressors on rewarding brain stimulation from the VTA is unavailable. It should be underscored that butyric acid and TMT effectively reduced ICSS performance (i.e. rate) at 80 Hz only. Examination of the data revealed that this decrement was most pronounced at the beginning of the descending curve and animals restored function at 80 Hz at the end of the frequency response curve. Such performance decrements were not associated with reward threshold shifts as measured by the frequency to maintain 50% of baseline ICSS responding

The influence of butyric acid and TMT on ICSS performance at 80 Hz was transient and may simply reflect a performance deficit that was restored upon brain stimulation. These data are hardly surprising given the powerfully rewarding influence of ICSS (Van Ree et al., 2000). The A10 area is considered a focal point of central reward neurocircuitry and a prominent DA/opioid interaction site. Wise (1989) has argued that the VTA is the neural interface for opiate reward neurocircuitry. Thus DA may play a primary or secondary role in mediating rewarding brain stimulation and the relative contribution of DA to reward may vary across brain regions (Wise, 1996). ICSS from the VTA in rats increases opioid peptide release from the VTA, nucleus accumbens, amygdala and prefrontal cortex (Stein, 1993). Interestingly, central administration of μ and δ opioid agonists increase operant responding for sucrose solutions in rats (Gosnell & Patel, 1993). Mu receptor density is conspicuous in

the VTA, δ receptor sites predominate in the nucleus accumbens and in the prefrontal cortex μ and δ are equally distributed (Mansour et al., 1995). Animals will self-administer enkephalin agonists into these sites indicating the rewarding properties and addictive potential of these substances (Devine & Wise, 1994; Wise, 1989). Naloxone attenuated self-administration of morphine into the amygdala and VTA of mice. However the rate of extinction was more rapid in the amygdala than in the VTA indicating the powerful motivational and/or rewarding effect of μ-receptor activation in the VTA (David & Cazala, 1994). In effect, the decrease in ICSS in mice exposed to butyric acid or TMT at 80 Hz was dependent on the temporal constraints of the frequency response curve. Mice in the butyric acid or TMT treatment group took longer to initiate ICSS performance presumably due to increased defensive scanning of the environment following stressor imposition.

In summary, the findings of the present series of experiments summarize the anxiogenic and anhedonic influence of the novel odor, butyric acid and the predator odor, TMT, on behaviour of CD-1 mice in the home-cage, light-dark box, acoustic startle and ICSS paradigms. The results of the present experimental set emphasize the importance of evaluating more than one response in order to gain a better understanding of the nature of fear, anxiety and hedonic processes following psychogenic stressor imposition. Both butyric acid and TMT induced anxiety in the light-dark box. However, TMT produced the strongest fear reactions among CD-1 mice. For example, exposure of CD-1 mice to TMT was associated with enhanced freezing and exaggerated anxiety in the light dark box. Both the indices of fear and anxiety in response to TMT application were associated with mesolimbic activation of ENK containing neurons. Moreover, TMT was more effective than butyric acid in enhancing

startle reactivity at protracted intervals. In contrast, both TMT and butyric acid were ineffective in inducing motivational changes as assessed by ICSS from the VTA presumably due to the highly rewarding properties of electrical stimulation of this mesolimbic site. It should be underscored that enkephalin release within the VTA, in particular, may be a neurochemically relevant cue in the initiation of motivation/reward threshold changes. In human and infrahuman subjects increased pro-enkephalin availability is associated with mood elevation and coping ability which may detract from the aversiveness of the stressor experience. In infrahuman subjects, activation of μ_1 and δ_1 receptors in the basolateral amygdala, VTA and mesocortex attenuates the aversiveness of psychogenic stressors. Taken together, mild stressors evoke gradations of anxiety dependent upon the motivational state of the animal.

EXPERIMENT 7

The putative influence of aversive life events to the provocation, maintenance and exacerbation of psychological disturbance is well documented (Anisman & Zacharko, 1990; Anisman & Zacharko, 1992; Brier, 1989; Bremner, 1999; Cui & Vaillant, 1997; Kessing et al., 1998; Kim & Yoon, 1998; Loas, 1996; Mazure, 1994; Risch, 1997; Weiss et al., 1999). In particular, there is considerable evidence to suggest that experience with aversive life events and the contextual cues associated with such experiences contribute to the provocation of behavioural disturbance among infrahuman subjects (Anisman et al., 1991). In rats, the expression of fear conditioning to stressor-associated cues appears within hours of association training, persists for at least 3 weeks and is resistant to prophylactic and therapeutic benzodiazepine intervention strategies (Ledoux, 1993, 1994). Nevertheless, initial exposure of

animals to the contextual cues associated with the light dark box (Chaouloff et al., 1997), the elevated plus maze (Griebel et al., 2000) or a predator odor (Barros et al., 2000; Dielenberg et al., 1999) induces anxiety which is ameliorated by benzodiazepine administration. However, the subsequent exposure of rats and mice to these identical paradigms effects an anxiety "form" which is resistant to the anxiolytic influence of either diazepam or midazolam (File & Zangrossi, 1993; McGregor & Dielenberg, 1999). In a similar vein, benzodiazepines are effective in ameliorating symptoms of generalized anxiety yet are relatively ineffective in panic disorder (Jonas & Cohen, 1993) and phobia (File et al., 1998; Marks, 1987; Tyrer, 1989). These data derived from infrahuman and human experimentation suggest that (a) divergent central sites and mesocorticolimbic regions, in particular, respond with differential sensitivity to stressors and anxiogenic events in particular (b) variable neurochemical activity underlies expression of behavioural disturbances associated with anxiety and (c) repeated encounters with anxiogenic stimuli provoke a pattern of neurochemical change consistent with the appearance of behavioural and neurochemical sensitization.

In humans, conditioned fear or fear-enhanced startle has been linked to psychological disorders which eventuate in sustained and exaggerated reactivity to environmental stressors. For example, an enhanced startle response following a catastrophic life experience has been linked to post-traumatic stress disorder (Morgan et al., 1995) and panic (Grillon et al., 1994). In rats and mice, the acoustic startle response is potentiated (e.g., sensitization) by unconditioned stimuli (e.g., light, tone), previously associated with footshock (e.g., fear-potentiated startle) (Brown et al., 1951; Falls et al., 1997). For example, an acute session of footshock (e.g., 1, 5, or 10 shocks, 500 msec duration, 0.2 -1.4 mA, 1 shock/second) increased startle to an acoustically associated cue 20-40 minutes following initial stressor application in

the rat (Davis, 1989). Moreover, exposure of animals to contextual cues (e.g., light) previously associated with a more severe stressor (e.g., 1 or 3 days of 40 tail shocks, 2 msec duration, 2 mA over 2 hours) also increased subsequent startle responsivity, albeit with a delayed onset (e.g., 7-10 days) (Servatius et al., 1995). Thus, the appearance of increased startle in response to stressor-associated cues was correlated with the intensity of the initial stressor experience including the frequency of shock imposition and number of stressor sessions employed (Davis et al., 1989; Servatius et al., 1995). In effect, neurogenic stressors may enhance the manifestation of behavioural features characteristic of severe anxiety conditions.

Protracted increases in startle reactivity have recently been demonstrated in humans following systemic CCK-4 administration (Shlik et al., 1999) and in rats following intraventricular (Frankland et al., 1996) and intra-amygdaloid administration of the CCK_B agonist pentagastrin (Frankland et al., 1997). Activation of CCK_B receptors by microiontophoretical and central administration of CCK-8S to the caudal pontine reticular nucleus (PnC), an amygdala projection site involved in the acoustic startle response of rats, enhanced acoustic startle (Fendt et al., 1995). Systemic administration of the CCK_B antagonist, L-365, 260, dose dependently reduced fear potentiated startle in rats previously exposed to mild footshock (0.5 mA, 0.5 second duration x 3 days) (Josselyn et al., 1995). Administration of alprazolam but not imipramine 30 minutes prior to testing dose dependently attenuated fear-potentiated startle in rats (Hijzen et al., 1995 c.f. imipramine, Cassella & Davis, 1985) and humans (Rodriguez-Fornells, 1999). The relative efficacy of alprazolam compared to imipramine in antagonizing fear potentiated startle in rats suggests that alprazolam may be more effective in influencing central sites underlying expression of

startle and conditioned behaviour, including enhanced CCK activity from the central nucleus of the amygdala (Davis & Shi, 1999; Harris & Westbrook, 1995).

The acoustic startle response (ASR) is a relatively simple response, characterized by rapid contraction (e.g. latency 5-10 msecs; duration 50-100 msecs) of facial and skeletal muscles following an unanticipated and intense auditory stimulus. The primary startle pathway includes only a few synaptic relays between cochlear nuclei, through a sensimotor interface (e.g., caudal pontine reticular nucleus) to muscles mediating the ASR (Koch & Schnitzler, 1997; Walker et al., 1997). Clinical investigations have demonstrated an enhanced startle amplitude (e.g., eye-blink and heart rate) and decreased startle latency in response to a startle probe (e.g., binaural burst of 110 dB white noise, 50 msec duration) previously associated with graphic photographic slides (e.g., wounds or mutilated bodies) in normal subjects who report high levels of basal anxiety (Cook et al., 1992; Grillon et al, 1991). In animals, prior emotional learning can modify the ASR. In rats, it has been reported that the association between an unconditioned stimulus (e.g. tone) and an aversive conditioned stimulus (e.g. light previously paired with footshock) induces physiological changes in neurons of the basolateral amygdala, such that the presence of the light enhances central amygdaloid modulation of caudal pontine reticular neurons (Davis et al., 1993). Increased startle latency, as well as, startle duration may be indicative of increased heterosynaptic input from the central nucleus of the amygdala to the pontine reticular nucleus following fear conditioning (Walker et al., 1997). It is conceivable that the anxiogenic influence of CCK_B receptor activation is occasioned by a neuronal enhancement of stressor saliency. In effect, the memory or recall of stressor-like experiences may be encoded by CCK variations in sub-areas of the amygdala (Flood et al., 1992).

The purpose of Experiment 7 was to assess the immediate (e.g. 30 minutes) and long-term (e.g., 1 week) effects of intraventricular administration of CCK-8S or systemic administration of the CCK_B agonist Boc CCK-4 on fear potentiated startle in CD-1 mice. Curiously, Boc CCK-4 is resistant to enzymatic degradation owing to its t-butoxycarbonyl N- terminal-protecting residue (Corringer et al., 1993; Deschodt-Lanckman et al., 1981). In addition, unlike CCK-8S, Boc CCK-4 is readily transported across the blood brain barrier and may be administered peripherally, but like CCK-8S, Boc CCK-4 decreased the number of entries into and time spent on the open arms of the elevated plus maze (Biro et al., 1997; Koks et al., 1999). However, systemic Boc CCK-4 is also associated with increased flight responses in rats in an animal model of panic (Mongeau & Marsden, 1997). There is suspicion that CCK_B receptor activation promotes protracted indices of anxiety or fear in individuals with panic and post-traumatic stress disorder (Bradwejn et al., 1990; Kellner et al., 1998) as well as infrahuman subjects relative to CCK-8S activation of both central CCK_A and CCK_B receptors (Fink et al., 1998; Mongeau & Marsden, 1997).

MATERIALS AND METHODS

Subjects and Surgery

Seventy, naive, male, CD-1 mice were group housed (12 weeks of age) with free access to food and water, and maintained on a 12 hour light dark schedule. Twenty-four mice were implanted with a 23-gauge cannula in the lateral ventricle (A.P. +0.8 mm from Bregma, L. +0.7 mm from midline and V. -2.7 to -2.9mm from a flat skull surface) under halothane anesthesia. Ventricular cannulation was accomplished with a David Kopf micromanipulator fitted with a mouse ventilator as previously described. Individual cannulae were fitted with a 30-gauge stylette. Following surgery, animals were housed individually, placed on a warm

heating pad and provided a dietary supplement (Meretene) for at least 3 days. Following postoperative recovery, mice were returned to the main animal area for at least 10 days prior to behavioural testing.

Drugs

Cholecystokinin-8 sulfated (CCK-8S) and N-t-Boc-Trp-Met-Asp-Phe-amide (Boc-CCK-4) (Sigma) were dissolved in 1 µl of DMSO and mixed with physiological saline. Intraventricular CCK-8S and vehicle injections were 1 µl in volume and occurred over a one-minute interval. The injector was left in place for an additional minute to ensure drug diffusion. Boc CCK-4 and vehicle injections were 0.6 ml in volume delivered subcutaneously in surgically naïve CD-1 mice.

Shock Apparatus

Uncontrollable footshock was administered in a 4.5 x 4.5 x 4.5 steel cage consisting of seven 3.0 mm diameter stainless steel bars spaced 6 mm apart through which shock could be administered. Shock intensity was set at 0.6 mA.

Startle Stimulus Pre-exposure and Baseline Startle Measures

The startle apparatus was previously described in Experiment 2. The procedure for eliciting fear-potentiated startle in mice followed the procedure outlined by Falls et al. (1997). In brief, mice were given 2 startle stimulus sessions separated by 24 hours in order to familiarize the mice with the startle apparatus and startle stimulus. The mice were placed in the startle cage and 5 minutes later presented with 10 startle stimulus trials at a 30-second interval. These data were not used in statistical analyses. Twenty-four hours later, mice were exposed to 3 startle sessions over 3 days. Mice were placed in the startle chamber and

assessed for startle reactivity with 10 startle stimuli at a variable interval of 30-35 seconds. The average of the 3 startle sessions over 3 days served as an indication of basal startle reactivity.

Training

Training was conducted in two sessions on 2 consecutive days. The mice were placed in the startle cage and 5 minutes later presented with 10 light + shock training trials consisting of a 30-s 75 W light that co-terminated with a 0.5-s, 0.6 mA footshock. The intertrial interval averaged 3.5 minutes and ranged from 2.5 to 4.5 minutes.

Post-training Tests of Fear-Potentiated Startle

Fear potentiated startle was assessed in mice (a) 30 minutes following systemic administration of 0 μg (n=10), 5 μg (n=10) or 15 μg (n=10) Boc CCK-4 or (b) immediately following intraventricular administration of 0 ng (n=8) or 50 ng (n=8) CCK-8S 24 hours after the last training day. The mice were placed in the startle cage and 5 minutes later presented with the light followed by the startle stimulus (Boc CCK-4 n=15; CCK-8S n=8) or the startle stimulus alone (Boc CCK-4 n=15; CCK-8S n=8) and assessed for startle reactivity over 10 sessions. A control group (Boc CCK-4 n=16; CCK-8S n=8) was included in which the mice were not given light and shock presentations and startle reactivity was assessed only to a startle stimulus following drug administration. To evaluate the persistence of fear-potentiated startle, mice were also tested 24, 48 and 168 hours following drug administration. Following behavioural testing, animals were perfused intracardially with physiological saline and 10% formalin. Brains were excised from the cranial cavity and placed in formalin for at least 2 weeks. The brains were subsequently blocked, frozen on a microtome and coronal sections

(40 μm) were mounted and stained with cresyl violet to verify cannula placement. Animals receiving systemic Boc CCK-4 drug administration were sacrificed with an overdose of CO_2.

Data Analysis

Startle amplitude, startle duration and latency to startle were converted to a percentage of Baseline. Behavioural percentage startle scores were subjected to analysis of variance (ANOVA) as a 2 (Drug: CCK or Saline) x 3 (Group: Tone and Tone + Light among previously shocked mice, Tone unconditioned among non-shocked mice) design with repeated measures over days (4). Separate analyses of variance were conducted for startle amplitude, startle duration and latency to startle in Boc CCK-4 and CCK-8S treatment groups.

RESULTS

Analyses of variance of the peak startle amplitude, startle duration or latency to startle associated with the various Startle conditions; Light + Tone (n=15) and Tone alone in shocked and non-shocked animals following 0 μg, 5 μg or 15 μg Boc CCK-4 administration failed to reveal any differences among treatment conditions (F's < 1, data not shown). Analyses of variance of the startle amplitude associated with the Light + Tone and Tone treatment conditions in shocked mice following systemic Boc CCK-4 administration at the various test intervals revealed a Drug x Group interaction, $F(2,24)=4.19$, $p<0.05$. Fisher's LSD multiple comparisons ($\alpha=.05$) revealed that the startle scores among Light + Tone exposed mice administered vehicle and 5 μg Boc CCK-4 were elevated relative to the scores among vehicle and 5 μg Boc CCK-4 treated mice in the Tone treatment condition. In addition, the startle scores of the Light + Tone mice challenged with 15 μg Boc CCK-4 were reduced

relative to mice administered 0 μg and 5 μg Boc CCK-4 under identical conditions (See Figure 7.1).

Insert Figure 7.1 about here

Analysis of variance of the startle amplitude associated with the Light + Tone and Tone treatment conditions following systemic Boc CCK-4 administration in previously shocked mice at the various test intervals examined also revealed a Day x Group interaction, $F(3,72)=2.79$, $p<0.05$. Fisher's LSD multiple comparisons ($\alpha=.05$) revealed that the startle scores of mice in the Light + Tone trials were elevated immediately, 24 and 168 hours following drug administration relative to mice exposed to the Tone alone (See Figure 7.2).

Insert Figure 7.2 about here

Analysis of variance of startle latency associated with the Light + Tone and Tone treatment conditions following systemic Boc CCK-4 administration in CD-1 mice at the various test intervals revealed a main effect of Group $F(2,24)=4.862$, $p<0.05$. Mice in the Light + Tone treatment condition displayed decreased startle latency relative to mice in the Tone treatment condition (See Figure 7.3).

Insert Figure 7.3 about here

Figure 7.1: Mean (± S.E.M.) startle amplitude (% baseline startle) among fear-conditioned mice following systemic saline, 5 μg or 15 μg Boc- CCK-4 administration. Saline or Boc-CCK-4 was administered 24 hours following the final day of fear conditioning. Testing was conducted with Light + Tone trials or Tone alone trials 30 minutes following injection. Startle values have been collapsed across test days. Note: * depicts a statistically significant difference ($p < .05$) among Tone and Light + Tone startle conditions in the identical drug condition.

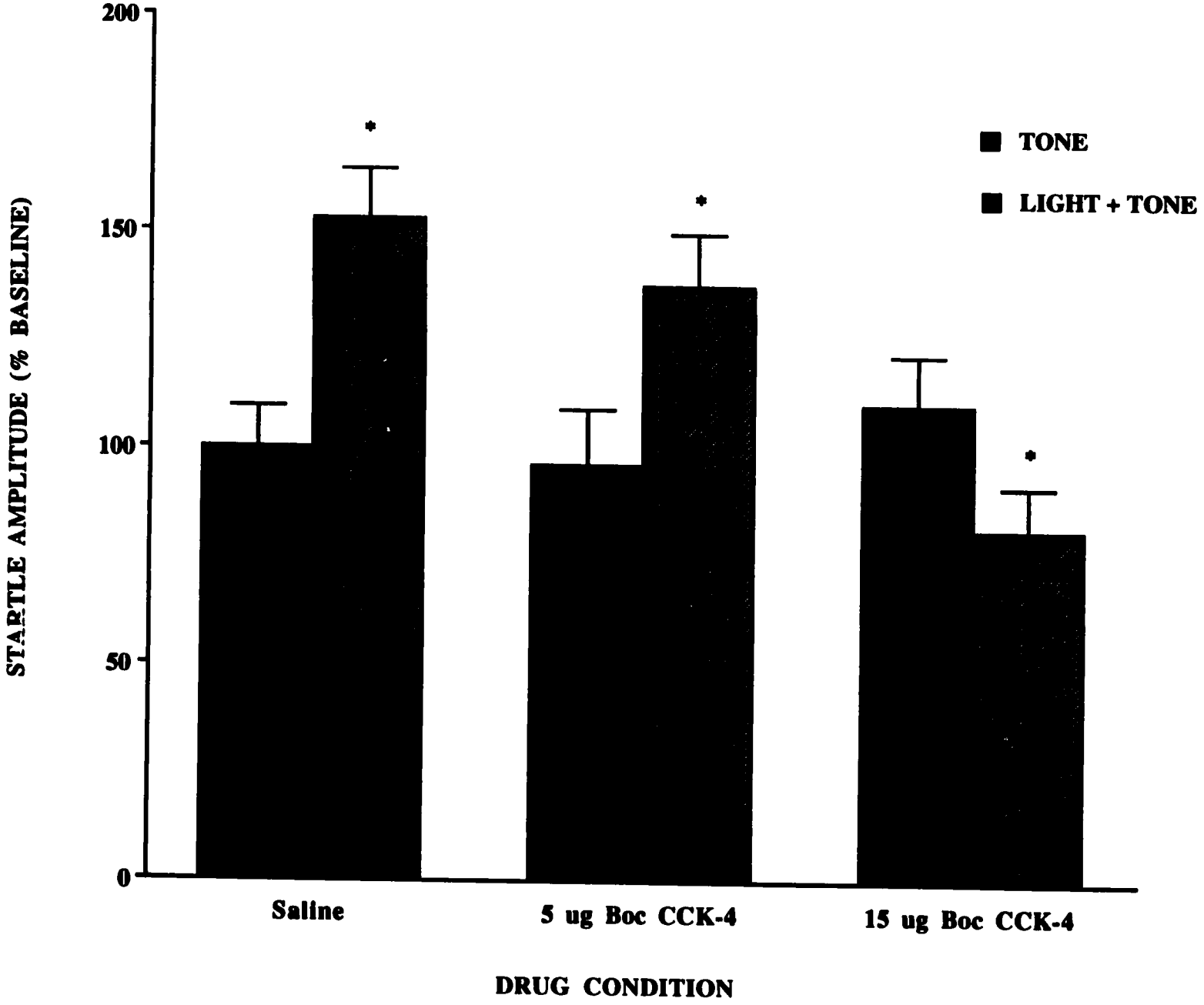

200
150
100
50
0
STARTLE AMPLITUDE (% BASELINE)
TONE
LIGHT + TONE
*
*
*
Saline
5 ug Boc CCK-4
15 ug Boc CCK-4
DRUG CONDITION

Figure 7.2: Mean (± S.E.M.) startle amplitude (% baseline startle) among fear-conditioned mice immediately (i.e., 30 minutes), 24 hours, 48 hours and 168 hours following drug administration in the Light + Tone or Tone alone treatment conditions. Startle values have been collapsed across drug dosages. Note: * depicts a statistically significant difference ($p < .05$) among Tone and Light + Tone startle conditions during the identical test interval.

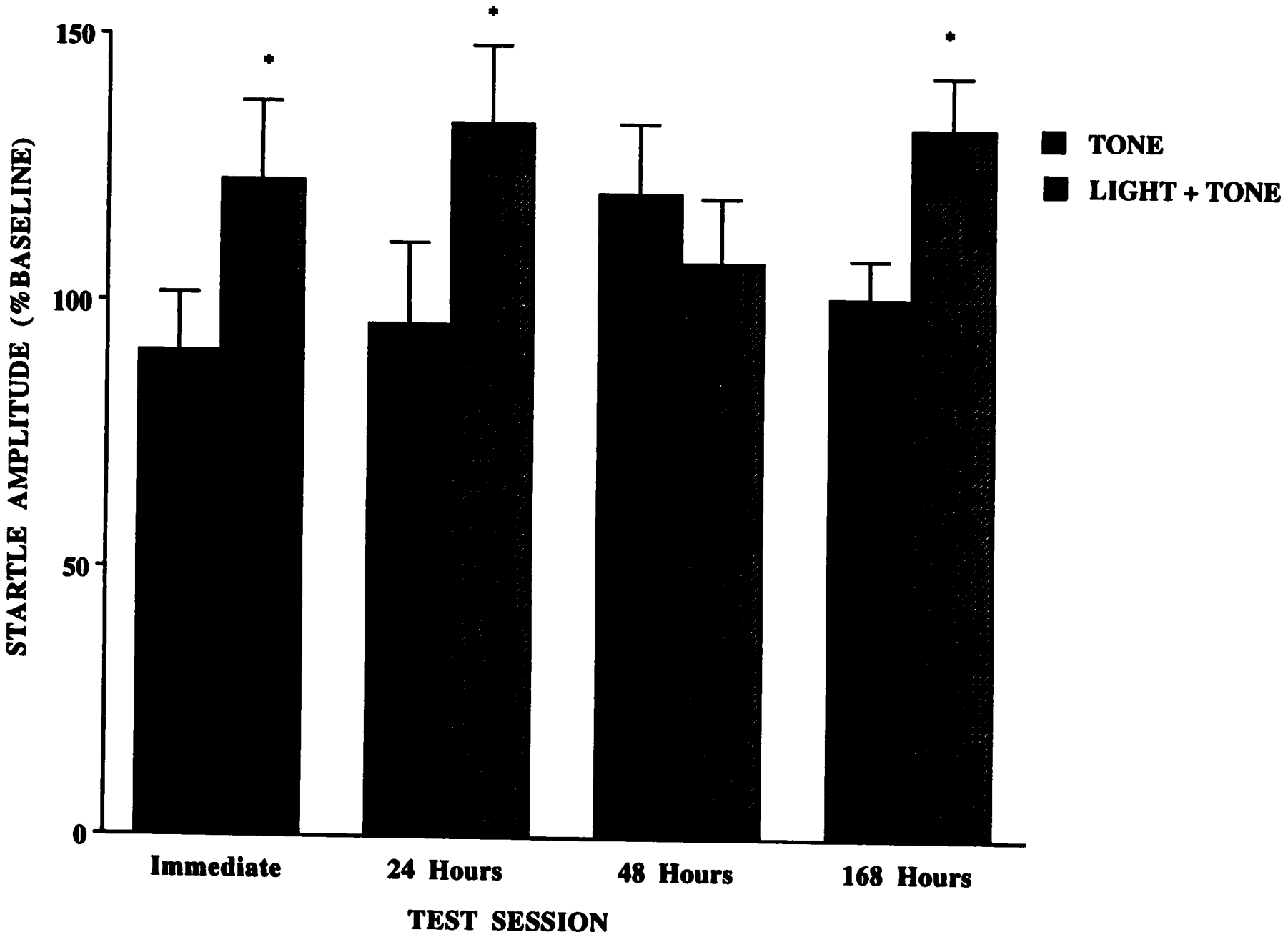
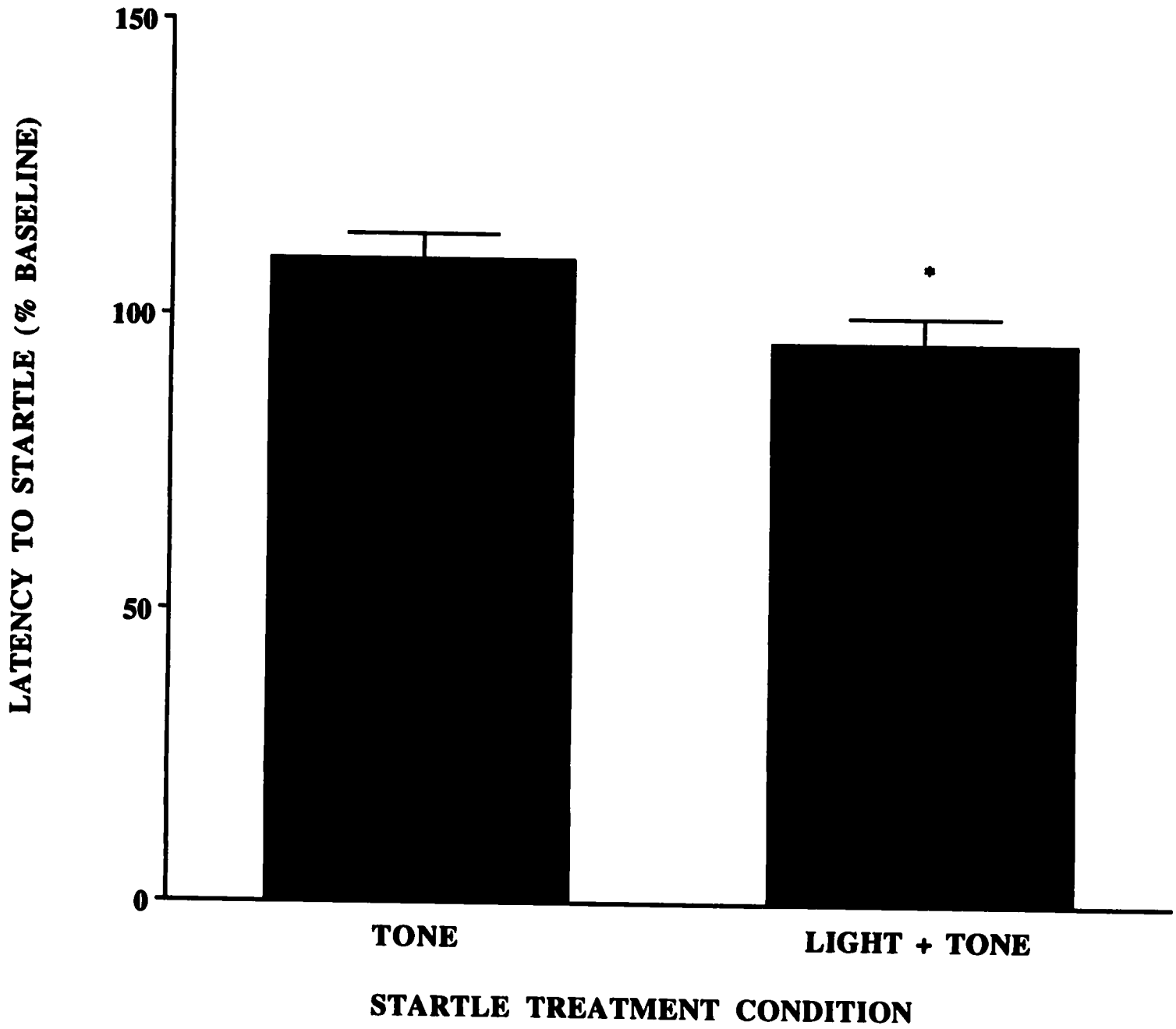

150
100
50
0
STARTLE AMPLITUDE (%BASELINE)
*
*
*
TONE
LIGHT + TONE
Immediate
24 Hours
48 Hours
168 Hours
TEST SESSION

Figure 7.3: Mean (± S.E.M.) startle latency (% baseline startle) among fear-conditioned mice following systemic saline, 5 μg or 15 μg Boc- CCK-4 administration in the Light + Tone or Tone alone treatment conditions. Startle latencies have been collapsed across drug dosages and test days. Note: * depicts a statistically significant decrease ($p < .05$) in latency to startle among mice in the Light + Tone startle condition relative to Tone alone.


150
100
50
0
LATENCY TO STARTLE (% BASELINE)
*
TONE
LIGHT + TONE
STARTLE TREATMENT CONDITION

Histological analyses verified cannula position in the lateral ventricle in animals that received either intraventricular saline or 50 ng CCK-8S. Analysis of variance of the peak startle amplitude associated with the various Startle conditions; Light + Tone (n=8) and Tone alone in conditioned (n=8) and non-conditioned (n=8) animals following intraventricular CCK-8S or saline administration revealed a main effect of Drug $F(1,18)=4.191$, $p<0.05$, Group $F(2,18)=10.486$, $p<0.001$ (see Figure 3.3), Day $F(3,54)=3.196$, $p<0.05$ and a Day x Drug interaction $F(3,54)=5.831$, $p<0.01$. Fisher's LSD multiple comparisons ($\alpha=.05$) revealed that the startle scores of mice in the Light + Tone trials were elevated relative to mice exposed to the Tone alone in conditioned and non-conditioned animals (See Figure 7.4).

Insert Figure 7.4 about here

Startle scores of mice in CCK-8S drug condition were enhanced relative to saline treated mice 48 and 168 hours following drug manipulation (See Figure 7.5).

Insert Figure 7.5 about here

Analyses of variance of the latency to startle (msec) associated with the various Startle conditions; Light + Tone (n=8) and Tone alone in shocked (conditioned) (n=8) and non-shocked (non-conditioned) (n=8) animals following intraventricular CCK-8S administration at the various test intervals revealed a main effect of Group $F(2,18)=11.030$, $p<0.001$, Day $F(3,54)=2.864$, $p<0.05$ and a Day x Group interaction $F(6,54)=3.070$, $p<0.01$.

Figure 7.4: Mean (± S.E.M.) startle amplitude (% baseline startle) among mice following intraventricular saline or 50 ng CCK-8S administration in the Light + Tone or Tone alone treatment conditions. Startle scores have been collapsed across drug dosages and test days. Note: * depicts a statistically significant increase ($p < .05$) in startle amplitude among mice in the Light + Tone startle condition relative to the Tone alone or the Unconditioned Tone.

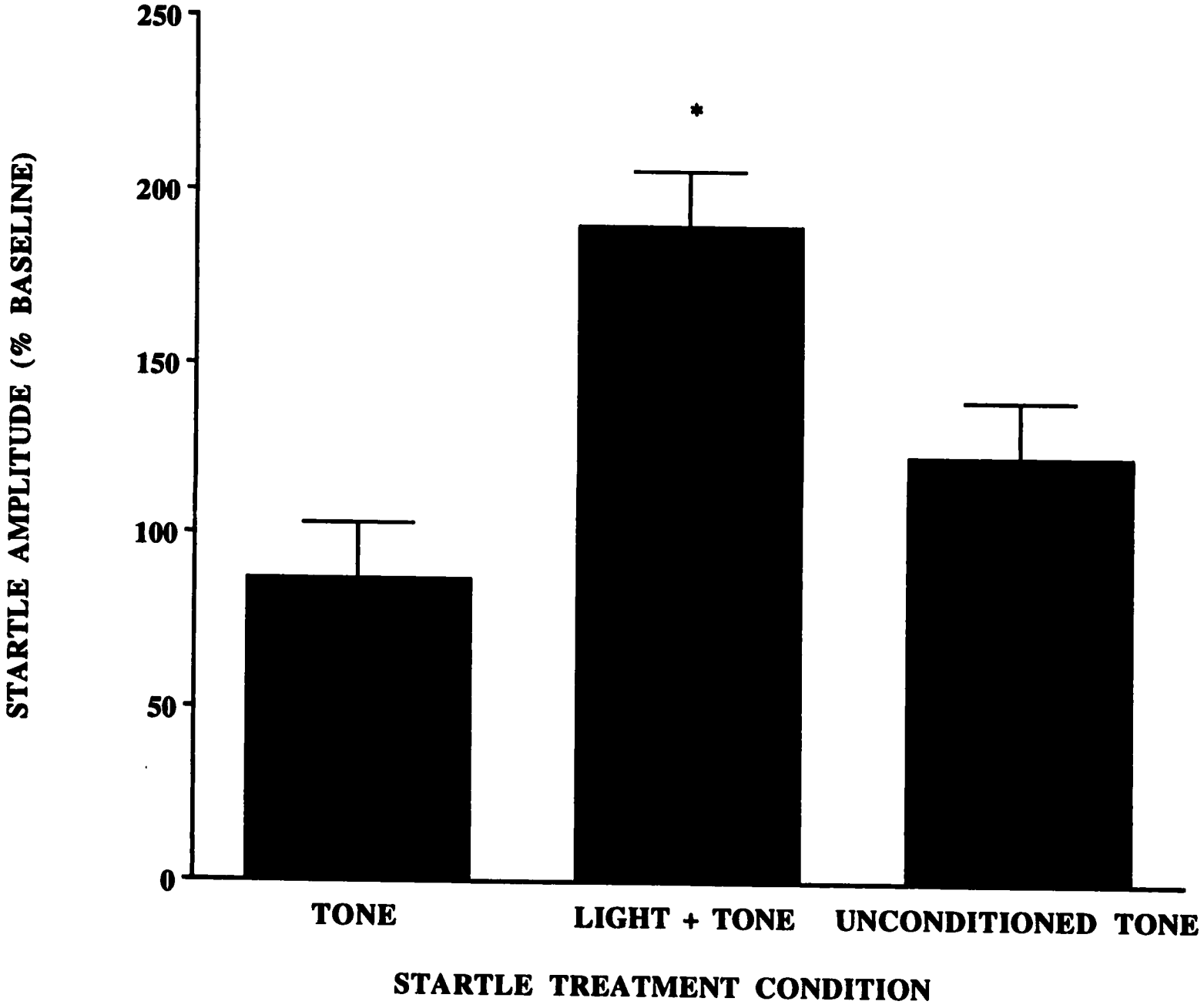
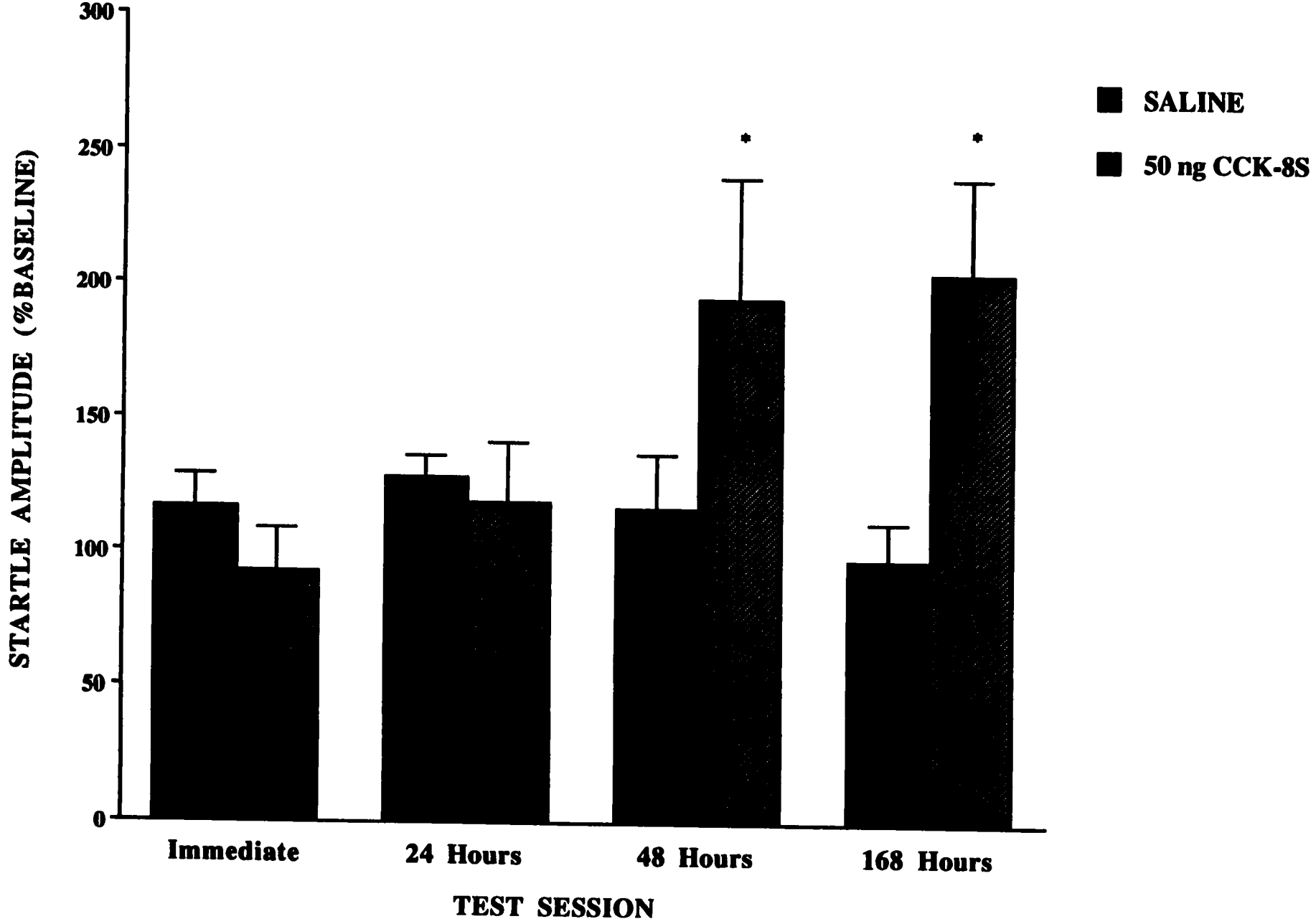

250
200
150
100
50
0
*
STARTLE AMPLITUDE (% BASELINE)
TONE
LIGHT + TONE
UNCONDITIONED TONE
STARTLE TREATMENT CONDITION

Figure 7.5: Mean (± S.E.M.) startle amplitude (% baseline startle) among mice immediately (i.e., 5 minutes), 24 hours, 48 hours and 168 hours following intraventricular saline or 50 ng CCK-8S administration. Startle values have been collapsed across Light + Tone or Tone alone treatment conditions. Note: * depicts a statistically significant increase ($p < .05$) in startle amplitude among mice administered 50 ng intraventricular CCK-8S relative to saline treatment 48 hours and 168 hours following drug manipulations.

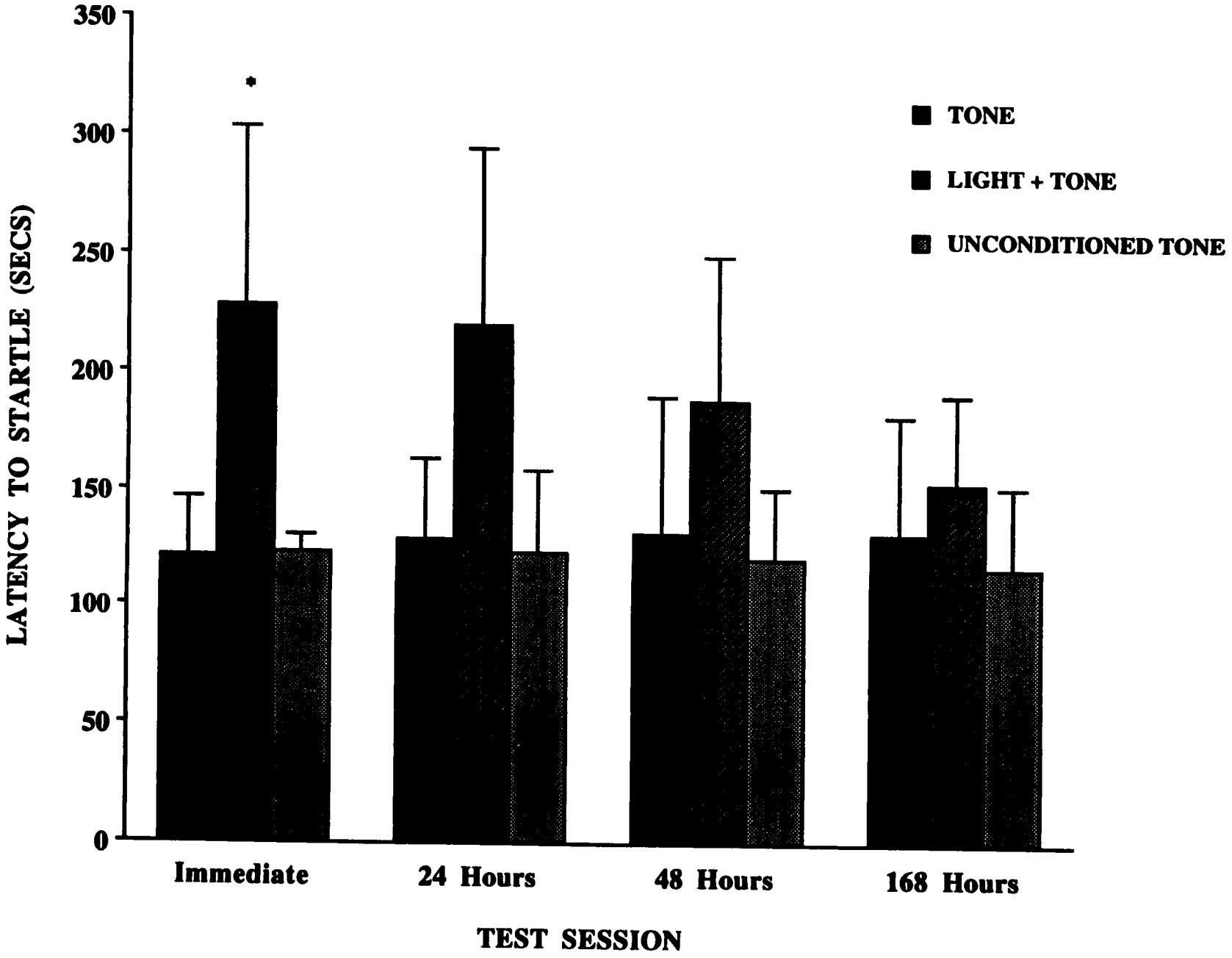

300
250
200
150
100
50
0
STARTLE AMPLITUDE (%BASELINE)
*
*
SALINE
50 ng CCK-8S
Immediate
24 Hours
48 Hours
168 Hours
TEST SESSION

Fisher's LSD multiple comparisons (α=.05) revealed that the latency scores of mice in the Light + Tone trials were elevated relative to mice exposed to the Tone alone in conditioned and non-conditioned animals during the immediate post-drug interval (See Figure 7.6).

Insert Figure 7.6 about here

Analyses of variance of the startle duration (msec) associated with the various Startle conditions; Light + Tone (n=8) and Tone alone in conditioned (n=8) and non-conditioned (n=8) animals treatment conditions following intraventricular CCK-8S administration at the various test intervals revealed a main effect of Group $F(2,18)=8.644$, $p<0.01$, Drug x Group interaction $F(2,18)=6.785$, $p<0.01$ and a Day x Drug x Group interaction $F(6,54)=2.595$, $p<0.05$ (see Figure 3.6). Fisher's LSD multiple comparisons (α=.05) of the simple simple main effects associated with the higher order interaction revealed that startle duration was decreased among CCK-8S treated mice relative to vehicle treated mice exposed to the Tone (conditioned) immediately, 24 hours and 168 hours post-drug administration. Startle duration was increased among CCK-8S treated mice relative to vehicle treated mice exposed to the Tone (unconditioned) immediately and 24 hours post-drug administration. Startle duration was decreased among CCK-8S treated mice relative to vehicle treated mice exposed to the Light + Tone 48 hours post-drug administration. Startle duration was increased among CCK-8S treated mice relative to vehicle treated mice exposed to the Light + Tone 168 hours post-drug administration. Startle duration of vehicle treated mice in the Light + Tone trials was elevated relative to mice exposed to the Tone alone (conditioned but not non-conditioned)

Figure 7.6: Mean (± S.E.M.) startle latency (% baseline startle) among mice following intraventricular saline or 50 ng CCK-8S administration in the Light + Tone or Tone alone treatment conditions. Startle latencies have been collapsed across drug dosages. Note: * depicts a statistically significant increase ($p < .05$) in startle latency among mice in the Light + Tone startle condition relative to the Tone alone or Unconditioned Tone startle condition.


350
300
250
200
150
100
50
0
LATENCY TO STARTLE (SECS)
*
TONE
LIGHT + TONE
UNCONDITIONED TONE
Immediate
24 Hours
48 Hours
168 Hours
TEST SESSION

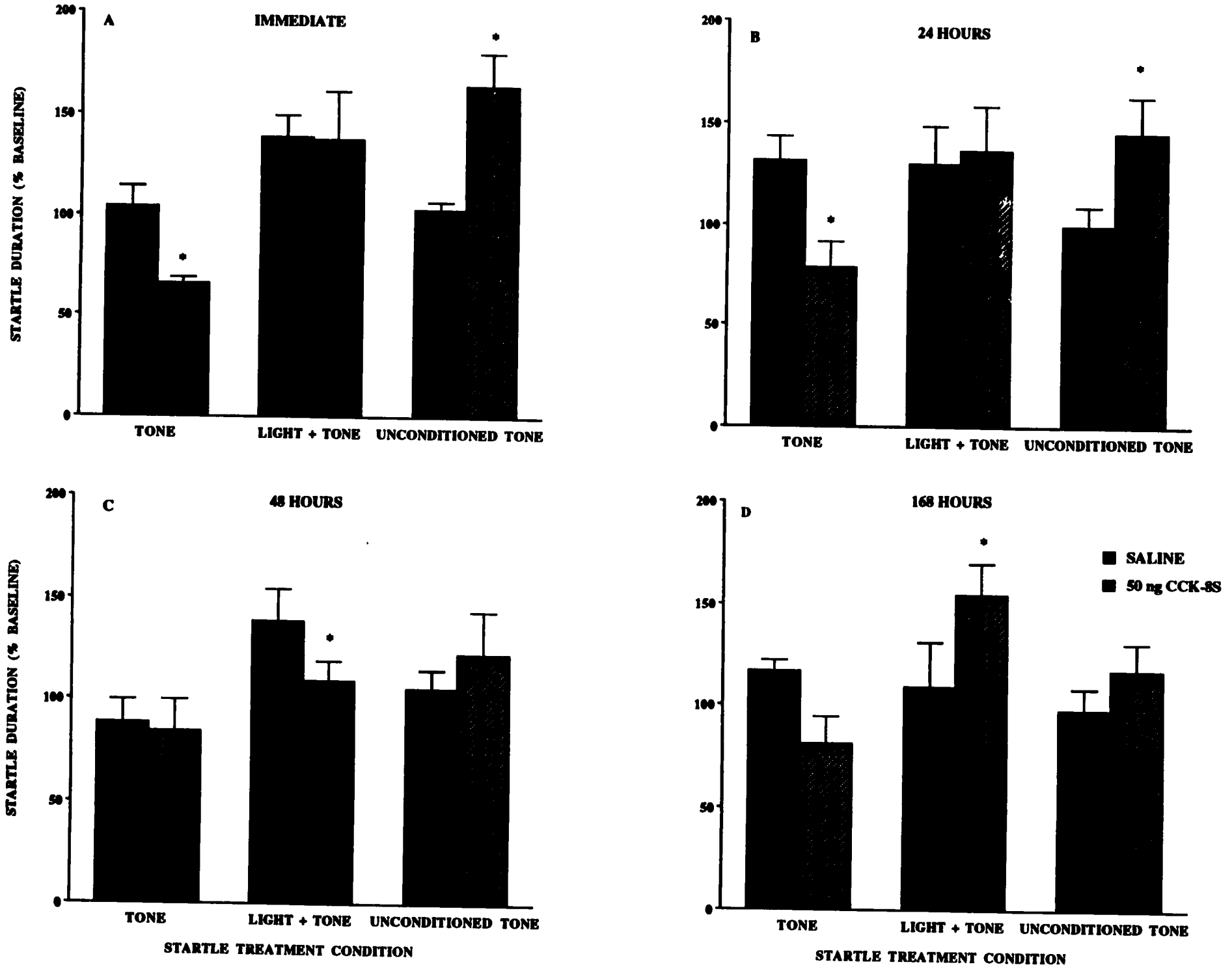
during the immediate and 48 hour post-drug intervals. Startle duration of CCK-8S treated mice in the Light + Tone condition was elevated relative to mice exposed to the Tone alone (conditioned but not non-conditioned) during the immediate, 24 hour and 168 hour post-drug intervals (See Figure 7.7).

Insert Figure 7.7 about here

DISCUSSION

The present findings of this investigation revealed that the acoustic startle reflex among 4-5 month old CD-1 mice is enhanced if a light previously paired with uncontrollable footshock is presented prior to startle stimulus onset. These data are consistent with data collected in several other laboratories employing similar paradigms in rats and several strains of mice (Falls et al., 1997; Walker et al., 1997). In particular, the results of the present investigation suggest that light previously paired with footshock (e.g. conditioned stimulus) induced a state of anticipatory anxiety in CD-1 mice, which was observed as an enhanced startle response, decreased latency of startle onset and increased startle duration. In keeping with this proposal, exaggerated startle amplitudes and increased startle duration may reflect increased startle magnitude while decreased startle latency may reflect reflex facilitation (Davis, 1992). The results of the present investigation revealed that among conditioned animals treated with Boc CCK-4 (0 μg, 5 μg, 15 μg) in the Light + Tone treatment condition decreased startle latency was evident relative to mice in the Tone treatment condition suggesting anticipation of aversive (i.e., startle) stimuli in the presence of Light (c.f. human

Figure 7.7: Mean (± S.E.M.) startle duration (% baseline startle) among mice (A) immediately (i.e., 5 minutes) (B) 24 hours , (C) 48 hours and (D) 168 hours following intraventricular saline or 50 ng CCK-8S administration in the Light + Tone or Tone alone treatment condition. Note: * depicts a statistically significant difference ($p < .05$) in startle duration among saline or CCK-8S treated mice in the identical startle condition and post-drug interval.


A
IMMEDIATE
B
24 HOURS
C
48 HOURS
D
168 HOURS
STARTLE DURATION (% BASELINE)
200
150
100
50
0
*
TONE
LIGHT + TONE
UNCONDITIONED TONE
STARTLE TREATMENT CONDITION
SALINE
50 ng CCK-8S

startle Grillon et al, 1991). Moreover, systemic administration of 5 μg of Boc CCK-4 potentiated startle amplitude in mice to the conditioned stimulus. The potentiated startle effect was maintained for 168 hours post-drug administration. Indeed, the difference in the startle scores of mice in the Light + Tone treatment condition relative to mice in the Tone alone group was most robust 168 hours following systemic Boc CCK-4 administration (i.e., cf. the immediate, 24 hours and 48 hours test intervals). However, the startle scores of mice within each treatment condition (i.e., Light + Tone, Tone) were collapsed across Boc CCK-4 drug doses and did not differ with respect to the day on which testing took place. Interestingly, when Test Days were collapsed, systemic administration of 5 μg Boc-CCK-4 was ineffective in altering the conditioned fear response relative to vehicle treated animals, however, potentiation of startle amplitude and decreased latency to startle were maintained.

In contrast, to the 5 μg dose of Boc-CCK-4 the 15 μg dose of Boc CCK-4 decreased startle reactivity among mice in the Light + Tone treatment group, while CD-1 mice exposed to the Tone alone exhibited startle scores comparable to vehicle treated animals under identical conditions. The decrease in startle reactivity in CD-1 mice in response to the 15 μg challenge dose of Boc CCK-4 was surprising and appear to contradict previously documented evidence pertaining to CCK_B receptor activation and startle reactivity among rats and mice. It will be recalled that systemic and intra-amygdaloid infusion of pentagastrin potentiated the acoustic startle reflex in rats (Frankland et al., 1996; 1997). Moreover, systemic administration of L-365, 260, dose dependently reduced fear potentiated startle in rats previously exposed to mild footshock (Josselyn et al., 1995). Clearly there is sufficient

evidence to suggest that activation of the CCK_B receptor promotes anxiety and/or fear related behaviour in startle paradigms.

The propensity of 15 μg of Boc-CCK-4 to attenuate and 5 μg to perpetuate acoustic startle may be related to the mnemotropic action of Boc CCK-4 and/or differential CCK_A/CCK_B activation of neurons in the startle circuit. For example, Gerhardt et al. (1994) demonstrated that systemic administration of Boc CCK-4 dose dependently accelerates habituation to environmental novelty in open field and hole-board paradigms (Gerhardt et al., 1994). Habituation is regarded as a form of learning that a repeatedly presented stimulus (i.e., startle stimulus) does not represent a biologically significant event and stimulus-evoked responding stops (Koch & Schnitzler, 1997). Avoidance learning was also enhanced among animals following central amygdaloid injection of Boc CCK-4 (Fekete et al., 1984; Huston et al., 1998). Interestingly, conditioned learning in rats is associated with CCK_A receptor activation (Josselyn et al., 1996). Indeed, the propensity of 15 μg Boc CCK-4 to attenuate startle reactivity may involve activation of CCK_A receptors within specific nuclei involved in the startle reflex. Indeed, Boc CCK-4 is not selective for the CCK_B receptor at high doses (Shiosaki et al., 1997) and interacts with CCK_A receptors owing to CCK_B receptor saturation (Fendt et al., 1995; Singewald & Sharp, 2000). Moreover, Fendt et al. (1995) reported that CCK_A and CCK_B receptors within the caudal pontine reticular nucleus are mutually antagonistic. In fact, CCK_B receptors in this central area are excitatory while CCK_A receptor activation is inhibitory (Fendt et al., 1995). In effect, an attenuation of startle would appear following CCK_A receptor activation.

Alternatively, the decrease in fear-potentiated startle among CD-1 mice following 15 µg Boc CCK-4 may be due to the relatively elevated shock amplitudes employed during fear conditioning. Davis and Astrachan (1978) suggested that acoustic startle in rats, for example, engenders an inverted U-shaped relationship to fear, such that intermediate shock intensities are optimal for fear conditioning. Interestingly, at such elevated shock intensities (e.g., high fear levels) the amygdala enhances dorsolateral periaqueductal gray neuronal activity, which in turn acts in an inhibitory fashion to attenuate startle (Walker et al., 1997). The behavioural correlate of such inhibition does not appear to coincide with immobility, although identification of the precise nature of this neural mechanism has yet to be elucidated. Nevertheless, dorsolateral periaqueductal gray neuronal activation induces defensive behaviours, including fight and flight, which are incompatible with the expression of fear-potentiated startle (Walker et al., 1997). It will be recalled that peripheral injection of Boc CCK-4 (40 µg) potentiated flight behaviour of rats in an animal model of panic (Mongeau & Marsden, 1997). However, it should be underscored that saline treated animals exhibited fear potentiated startle. The relative shock intensity, by itself, therefore does not account for the decrease in startle reactivity among CD-1 mice challenged with 15 µg Boc CCK-4. Indeed, Davis (1992) maintained that as shocks are withheld during testing, drug effects couldn't be explained by alterations in shock sensitivity. Moreover, the 0.6 mA shock intensity used in the present experiment produced maximal fear conditioning in rats and several strains of mice (Falls et al., 1997; Walker et al., 1997).

It might be noted incidentally that uncontrollable footshock has been associated with CCK release from mesolimbic sites (Siegel et al., 1984) including the amygdala which contains

a high abundance of CCK_B receptors (Hill & Woodruff, 1990). In the present investigation, 15 μg Boc CCK-4 in previously stressed animals may induce an anxiety state, inconducive to fear conditioning. This is supported by the present data. The lower dose of Boc CCK-4 (5 μg) but not the higher dose (15 μg) of Boc-CCK-4 enhanced startle in mice previously exposed to footshock. Pilot data collected in this laboratory revealed that non-shocked mice administered 5 μg or 15 μg Boc-CCK-4 do not exhibit increased acoustic startle responses. In fact, when this group of animals was included in statistical analyses (Unconditioned Tone) no differences among treatment conditions were noted. Taken together, the interplay between doses of CCK administered and resultant anxiety severity, recruitment of neural fibers involved in the potentiation (i.e., amygdala) or inhibition (i.e., periaqueductal gray) of conditioned fear and/or the saliency of stressor-associated cues may modulate startle responsivity.

In the present experiment, intraventricular administration of 50 ng CCK-8S collapsed across treatment conditions (i.e., Light + Tone, Tone) enhanced startle amplitude 48 hours and 168 hours post-drug manipulation. These data suggest that exogenous CCK-8S application influences startle amplitude independently of prior stressor history. Intraventricular administration of CCK-8S dose dependently (0, 5 ng, 25 ng and 50 ng) increased anxiety in the light-dark box immediately following drug administration in non-shocked CD-1 mice (MacNeil et al., 1997). It will be recalled that startle stimuli themselves are aversive and may induce protracted states of fear or anxiety (Borszcz et al., 1989). Interestingly, the pontine reticular nucleus projects onto cranial and spinal neurons which are responsive to relatively intense acoustic stimulation (e.g., 75 dB) (Koch & Schnitzler, 1997).

CCK-8S increased latency of startle onset in the Light + Tone condition in the immediate post-drug interval. However, CCK-8S had variable influence on startle duration depending on the prior stressor history of the animal. CCK-8S decreased startle duration in the Tone condition of previously shocked mice immediately, 24 hours and 168 hours post-drug administration relative to saline treated mice. Among non-shocked mice, CCK-8S increased startle duration in the Tone condition immediately, 24 hours and 168 hours post-drug administration relative to saline treated mice. CCK-8S decreased and increased startle duration in the Light + Tone condition 48 hours and 168 hours post-drug administration relative to saline treated mice, respectively. The increase in startle latency and duration following CCK-8S administration in Light + Tone group may be indicative of heterosynaptic input from the central nucleus of the amygdala to the pontine reticular nucleus following fear conditioning (Walker et al., 1997). It should be underscored that although CCK-8S stimulates both CCK_A and CCK_B receptors, receptor binding studies reveal that CCK-8S has a greater affinity for CCK_B receptors (Harhammer et al., 1991). Fendt et al. (1995) demonstrated that microiontophoretic administration of CCK-8S in the pontine reticular nucleus dose-dependently increased the acoustic startle response in rats. Moreover, CCK locally administered into the pontine reticular nucleus (Fendt et al., 1995) or the amygdala (Frankland et al., 1997) produced protracted increases in ASR indicating that CCK is involved in sensitization. The neural mechanisms underlying the variable influence of CCK-8S on startle parameters remain to be elucidated but CCK amygdaloid activation cannot be dismissed.

In humans and animals the amygdala appears to participate in the acquisition and retention of emotionally laden stimuli (Blanchard & Blanchard, 1972; Davidson & Irwin, 1999). Such central organicity has been linked to deficits in learning and memory (Maes et al.,

1998; Post et al., 1998) and increased startle reactivity (Allen et al., 1999). Indeed, the amygdala is a critical area of plasticity that mediates both the acquisition and extinction of conditioned fear and anxiety (Davis, 1992). In rats, lidocaine associated inactivation or electrolytic lesions of the basolateral nucleus of the amygdala prevented consolidation of the emotional learning and conditioning of acoustic startle (Gewirtz et al., 1998). Infusion of the AMPA receptor antagonist NBQX into the central amygdaloid nucleus (Walker & Davis, 1997) or the NMDA receptor antagonist, AP5, into the basolateral amygdaloid nucleus (Gewirtz & Davis, 1997) of the rat blocked fear potentiated startle to a cue previously paired with footshock. These data propose an NMDA-dependent form of neural plasticity (e.g. long-term potentiation) occurs in the basolateral and central amygdaloid nuclei during fear conditioning. This plasticity may result in long-term changes that are elicited by the acquisition of emotions to specific salient environmental cues. Taken together, sub-areas of nuclei within the acoustic startle pathway may be sensitive to diverse aspects of the stressor as well as conditioned stimulus. Moreover, CCK_A/CCK_B receptor activation within these sites coordinates specific emotional reactivity (e.g. startle) and cognitive responses, including increased memory of and/or sustaining attention to mild stressors which may modulate the overt expression of anxiety and fear. In summary, it is likely that the prophylactic and therapeutic treatment of anxiety disorders will engender the use of selective CCK_B antagonists as more effective management strategies.

EXPERIMENTS 8 AND 9

It is well documented that stressful life experiences contribute to the etiology of human mood disorders, including anxiety, depression and panic (Post et al., 1998). In fact, it has been reported that while recurrent anxiety episodes provoke anhedonic episodes repeated

anxiety together with the affective disturbance of depression may evoke severe anxiety conditions including panic (see Keller & Hanks, 1993 for review). A role for CCK in the induction of anxiety in infrahuman subjects (Cohen et al., 1998), promotion of panic (Bradwejn et al., 1990) and co-expression of severe depression and anxiety in humans (Lofberg et al., 1998) appears to be conspicuous. In rats rated anxious with respect to performance in the elevated plus maze, a reduced benzodiazepine receptor density and increased CCK-8S binding in the frontal cortex was in evidence relative to non-anxious counter-parts (Harro et al, 1990). Chronic benzodiazepine treatment in rats decreases neuronal responsivity to microiontophoretically applied CCK-8S in the frontal cortex and hippocampus (Bouthillier & DeMontigny, 1988; Bradwejn & DeMontigny, 1984) suggesting benzodiazepines suppress CCK-8S activation in the prefrontal cortex of anxious mice (Harro & Vasar, 1991). Central administration of CCK-8S in infrahuman subjects has been associated with anxiety in the elevated plus maze (Biro et al., 1997; Johnson & Rodgers, 1996; Ravard & Dourish, 1990) and light-dark paradigms (MacNeil et al., 1997), increased threshold for previously rewarding brain stimulation from the rat nucleus accumbens (Heidbreder et al., 1992) and VTA (Singh et al., 1997; Vaccarino & Koob, 1984) and suppressed locomotor activity in mice (Hirosue et al., 1992) reminiscent of uncontrollable footshock (Hebb et al, 1997; Zacharko et al, 1990; Zacharko et al, 1998). At the very least, these data suggest that increased CCK availability incites anxiety and motivational alterations among infrahuman and human subjects.

It will be recalled that CCK-8S is colocalized with DA in the mesencephalon, nucleus accumbens, mesocortex as well as the central amygdaloid nucleus in rats (Bunney, 1987; Harris & Nestler, 1996; Hokfelt et al., 1980; Hokfelt et al, 1994; Lundberg & Hokfelt, 1983;

Palacios et al., 1989; Seroogy et al., 1989; Studler et al., 1982; Studler et al., 1984; Voigt et al., 1986). Indeed, mesencephalic DA and CCK have been identified in the same presynaptic vesicle (Studler et al., 1984). Cholecystokinin appears to modulate DA release in mesocorticolimbic and nigrostriatal sites and to regulate the electrophysiological activity of DA neurons (Jackson & Westlind-Danielson, 1994). Increased threshold for previously rewarding brain stimulation from the VTA as well as suppressed locomotor activity following central CCK-8S administration has been linked to activation of CCK_B receptors and subsequent decreased DA activity in the VTA and nucleus accumbens, respectively (Crawley, 1988; Crawley, 1992; Fiorino et al, 1993; Marshall et al, 1991; Singh et al, 1997). Exposure of animals to a variety of psychogenic (e.g., predator odors) and neurogenic (e.g., footshock) stressors promotes corelease of mesocorticolimbic DA (Cabib et al, 1988; Deutch et al, 1985; Imperato et al, 1989; Keefe et al, 1990; Saavedra, 1982; Watanabe, 1984) and CCK (Harro et al, 1996; Siegel et al, 1987; Pavlasevic et al, 1993). This observation is consistent with previous hypotheses that the circumstances defining corelease of DA and CCK may contribute to the expression of specific features of some psychological illness (Hokfelt et al, 1980), including psychomotor retardation, anxiety (Pitchot et al., 1992; Roy-Byrne et al., 1986), melancholia (Roy et al., 1985) and anhedonia (Markou & Koob, 1991).

It should be considered that CCK release following stressor imposition decreases DA activity by reducing enkephalin availability in the nucleus accumbens, VTA and mesocortex (Curran & Watson, 1995; Savasta et al., 1989). The antagonistic effects of CCK-8S on opiate associated analgesia (Noble et al., 1994; 1996 for review) and locomotor activity (Bourin et al., 1999) in rats have been documented as evidence that CCK is an opioid receptor antagonist. However, there is a relative paucity of information which characterizes the role of

CCK and enkephalin in animal models of anxiety and depression. Indeed, it is conceivable that a CCK-enkephalin interface in mesolimbic sites may alter expression of motivation and affect. Such an analysis would impact on the description of stressor effects, in particular the parameters associated with stressor-induced CCK release and the propensity of enkephalin to blunt the motivational and affective alterations attending such experiences.

It is well documented that endogenous opioids contribute to the expression of affect and motivation in human (Castilla-Cortazar et al., 1998; Cohen et al., 1984; Zis et al., 1985) and infrahuman subjects (Hernandez et al., 1997). Indeed, major affective disorder has been associated with decreased cerebrospinal (CSF) endorphin concentrations (Berger & Nemeroff, 1987; Djurovic et al., 1999). Likewise, among infrahuman subjects enkephalinase inhibitors possess antidepressant properties (Tejedor-Real et al., 1998), the therapeutic efficacy of various antidepressant agents are attenuated by naloxone (DeFelipe et al., 1985, 1989) and chronic antidepressant administration increases central enkephalin availability in the mesencephalon (Dziedzicka-Wasylewska & Papp, 1996). Met- and leu-enkephalin are readily self administered into the VTA and clearly support conditioned place preference (McBride et al., 1999). The conjoint activation of both the μ and the δ receptor augments mesencephalic self-stimulation and promotes locomotor activity and rearing among stressed (Hebb et al., 1997; Zacharko et al., 1998) and non-stressed (Heidbreder et al., 1992) mice. The A10 area is considered a focal point of central reward neurocircuitry and a prominent DA/opioid and DA/CCK interaction site. Wise (1989) has argued that the VTA is the neural interface for opiate reward neurocircuitry. Thus DA may play a primary or secondary role in mediating rewarding brain stimulation (Fiorino et al, 1993; Nakajima & Patterson, 1997). In effect, the

relative contribution of DA to reward threshold changes following central CCK administration may vary across brain regions Taken together, these data suggest that μ and δ receptor activation may attenuate CCK-8S-induced anhedonia and concomitant increases in reward threshold in mice responding for brain stimulation from the VTA as well as abrogate CCK-induced behavioural deficits including locomotion and rearing.

Experiment 8 evaluated the immediate influence of a 50 ng dose of CCK-8S on threshold changes for previously rewarding brain stimulation from the dorsal and ventral aspects of the VTA. It should be underscored that it has been previously been demonstrated in this laboratory that brain stimulation from ventral aspects of the VTA is stressor resistant (Hebb et al., 1997; Zacharko et al., 1998). The long-term evaluation of an acute intraventricular CCK-8S administration on brain stimulation from the dorsal aspects of the VTA in CD-1 mice was extended to (a) consider the therapeutic efficacy of the peptidase resistant enkephalin analogue with mixed μ/δ receptor agonist properties, D-Ala^2-Met^5-enkephalinamide (DALA) in ameliorating putative anhedonic effects immediately and for one week following 50 ng CCK administration and (b) evaluate the prophylactic influence of DALA intervention to the attenuation of CCK associated re-induction of anhedonia to a previously established sterile anxiogenic CCK dose (i.e., 5 ng) given 18 days (or 11 days following the last ICSS session at 7 days) following 50 ng CCK-8S. The temporal schedule for central CCK-8S and DALA administration, as well as CCK reexposure, were predicated on data recently collected in this laboratory which revealed central administration of the enkephalin analogue DPDPE selective for the δ receptor immediately following uncontrollable footshock and novelty in CD-1 mice increased (rather than decreased)

locomotor activity with reexposure to only the identical, milder form of the original stressor. In this regard, Experiment 9 examined the same schedules of CCK-8S and DALA administration and CCK re-exposure as Experiment 8 on locomotor and rearing behaviour in CD-1 mice.

MATERIALS AND METHODS

Subjects and Surgery

Naive, male, CD-1, mice (Experiment 8, N=52; Experiment 9, N=37) were anesthetized with halothane and stereotaxically implanted with a bipolar stainless steel electrode (0.5 mm tip to tip, Plastic One, Roanoke, Va.) in the mesencephalon and a cannula (23 gauge) in the lateral ventricle (Experiment 8) or only a lateral ventricle cannula (Experiment 9) as described in Experiments 6 and 7. Stereotaxic coordinates for the ventral A10 electrode placements were A.P. -3.0 to -3.2 mm from Bregma, L. +0.5 mm from the sagittal suture and V. -5.2 mm from flat skull surface.

Drugs

Cholecystokinin-8 sulfated was prepared as described in Experiment 7. D-Ala^2-Met^5-enkephalinamide (DALA) (Sigma) was dissolved in physiological saline.

Histology

Histological verification procedures for electrode and cannula placements were previously described in Experiments 6 and 7.

Experiment 8

Self-Stimulation Training

The self-stimulation chambers and training were previously described in Experiment 6 with the exception of the following details. Following completion of the descending phase of

the test session, brain stimulation frequencies were increased by 10 Hz steps starting again from 20 Hz increasing in 10 Hz steps at 3-minute intervals to 80 Hz. Mice were then removed from the self-stimulation tubs and handled in such a way as to simulate intraventricular drug administration. Mice were then returned to the tubs for a subsequent 42 minutes session. Self-stimulation performance was averaged across the descending and ascending frequency modes for both 42 minute sessions following stable responding (<10% variation on three successive days) and a response curve was established for each animal according to previously established protocols (Fibiger & Phillips, 1981). The scores of all animals were subsequently averaged for the respective treatment conditions and electrode placement in the dorsal or ventral aspects of the VTA.

Following determination of baseline self-stimulation performance, mice in each of the dorsal (N = 40/ n= 5 animals per 8 treatment groups) and ventral (N = 12/Saline n=6 50 ng CCK-8S n=6 animals per 2 treatment groups) A10 conditions received either 50 ng of cholecystokinin-8 sulfated (CCK-8S) (n=20) or physiological saline (n=20; Day 1; Drug 1), intraventricularly. Following injection, the stylette was reinserted and the animal was immediately returned to the self-stimulation chambers and tested for self-stimulation. Frequency response curves were again generated and the frequency of brain stimulation from the dorsal aspects of the VTA that approximated half-maximal performance at 80 Hz was determined for each animal in intraventricular saline or CCK conditions. The use of the half-maximal procedure was obtained from previously established criteria pertaining to the rewarding value of brain stimulation (Milliaressis et al, 1986) and has been employed in laboratories using both current intensity (Kokkinidis & McCarter, 1990) and frequency (Nakajima & O'Regan, 1991). In the case where mice failed to reach 50 % ICSS responding

at 80 Hz following CCK-8S administration (i.e., 50 ng CCK-8S) 80 Hz was arbitrarily adopted as the reward frequency for statistical analysis. Mice with electrodes in the dorsal aspects of the VTA were then removed from the tubs and given 1.0 μg of DALA (n=20) or physiological saline (n=20) (Drug 2) and reassessed for self-stimulation performance immediately (Day 1), 24 hours (Day 2), 48 hours (Day 3) and 168 hours (Day 7) following initial drug injections. The frequency of brain stimulation that approximated half-maximal performance at 80 Hz was determined for every animal in each of the 4 treatment conditions (SAL/SAL; SAL/CCK; CCK/SAL; CCK/DAL) for each of the 4 test sessions. Eighteen days following initial drug treatment (or 11 days following 168h test) mice received either 5 ng CCK-8S (n=20) or physiological saline (n=20) (Drug 3). Mice were assessed for self-stimulation performance immediately (Day 18), 24 hours (Day 19), 48 hours (Day 20) and 168 hours (Day 24). Finally, the reward threshold frequency was determined for every animal in each of the 8 treatment conditions (n=5 per 8 treatment conditions) over the 4 test sessions. Again, in the case where mice failed to reach 50 % ICSS responding at 80 Hz (i.e., CCK/SAL/CCK), the 80 Hz frequency was adopted for statistical purposes. Similarly, in the case where mice exceeded 50 % ICSS responding at 20 Hz (i.e., CCK/DALA/CCK), the 20 Hz frequency was adopted as the reward threshold. Drug injections occurred on Day 1 (50 ng CCK-8S/Saline; 1μg DALA/Saline) and Day 18 (5 ng CCK-8S/Saline), only.

Data Analysis

All ICSS frequency response scores from animals with electrodes in the dorsal or ventral aspects of the VTA were converted to a percentage of maximum Baseline (80 Hz). Separate analyses of variance (ANOVA) were conducted for animals with electrodes in the

dorsal versus ventral aspects of the VTA. Animals with electrodes in the ventral aspects of the VTA received only a single injection of either CCK or Saline on Day 1 based on a priori hypothesis that CCK-8S would not induce decrements in self-stimulation performance or increase reward thresholds. ICSS percent baseline scores among mice responding for brain stimulation from the ventral aspects of the VTA were subjected to a one-way ANOVA (CCK or Saline) with repeated measures over frequencies. ICSS percent baseline scores among mice responding for brain stimulation from the dorsal aspects of the VTA were subjected to ANOVA as a 2 (CCK or Saline; Drug 1) x 2 (DALA or Saline; Drug 2) x 2 (CCK or Saline; Drug 3) design with repeated measures over frequencies (7) and test sessions (9). An additional ANOVA for detection of frequency reward threshold changes among mice with electrodes in the dorsal aspects of the VTA was conducted as a 2 (CCK or Saline; Drug 1) x 2 (DALA or Saline; Drug 2) x 2 (CCK or Saline; Drug 3) design with repeated measures over test sessions (9). Fisher's least significant difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

RESULTS

Histological analyses verified cannula position in the lateral ventricle and electrode placement in the dorsal or ventral aspects of the VTA. Six animals were not included in the statistical analyses due to electrode placements in nuclei other than the dorsal (n=4) or ventral sub-regions (n=2) of the VTA and 5 mice were not included the statistical analyses due to cannulae positioned outside of the lateral ventricle (n=3), severe tissue necrosis (n=1) or drug diffusion (n=1). Schematic representations of the distribution of dorsal and ventral A10 electrode positions are presented in Figure 8.1.

Insert Figure 8.1 about here

Analysis of variance revealed that baseline frequency response curves among mice responding for brain stimulation from the dorsal or ventral aspects of the VTA in the vehicle and 50 ng CCK-8S conditions did not differ from one another, $F(1, 16)= 0.15$, $p>.05$). As expected, baseline self-stimulation performance from the dorsal and ventral aspects of the VTA varied with stimulation Frequency $F(6, 96)= 43.538$, $p<. 001$, such that increments in self-stimulation performance were detected as self-stimulation frequency increased. The baseline performance of mice responding for brain stimulation from the dorsal and ventral aspects of the VTA were expressed as a percentage of maximal baseline performance achieved at 80 Hz (see Figure 8.2).

Insert Figure 8.2 about here

Analysis of variance of baseline frequency response curves of animals responding for brain stimulation from the ventral aspects of the VTA revealed a significant effect of Frequency $F(6, 48)= 32.497$, $p<. 0001$) yet failed to reveal a significant difference among mice assigned to either saline or CCK treatment groups prior to drug injection, $F(1, 8)= .263$, $p>.05$. Predictably, the baseline self-stimulation performance of mice with electrodes in the ventral aspects of the VTA increased with increasing stimulation frequency (see Figure 8.3).

Insert Figure 8.3 about here

Figure 8.1: Schematic representation (rostral-caudal from Bregma) depicting electrode placements in the dorsal and ventral aspects of the VTA among mice treated intraventricularly with saline or 50 ng CCK-8S. In the dorsal VTA, open circles denote Saline treated mice and closed circles represent CCK treated mice. In the ventral VTA, open squares denote Saline treated mice and closed squares represent CCK treated mice. Brain areas identified include the ventral tegmental area (VTA), substantia nigra (SN) and the interpeduncular nucleus (IP). There is overlap of electrode position in the AP plane among the respective treatment conditions.

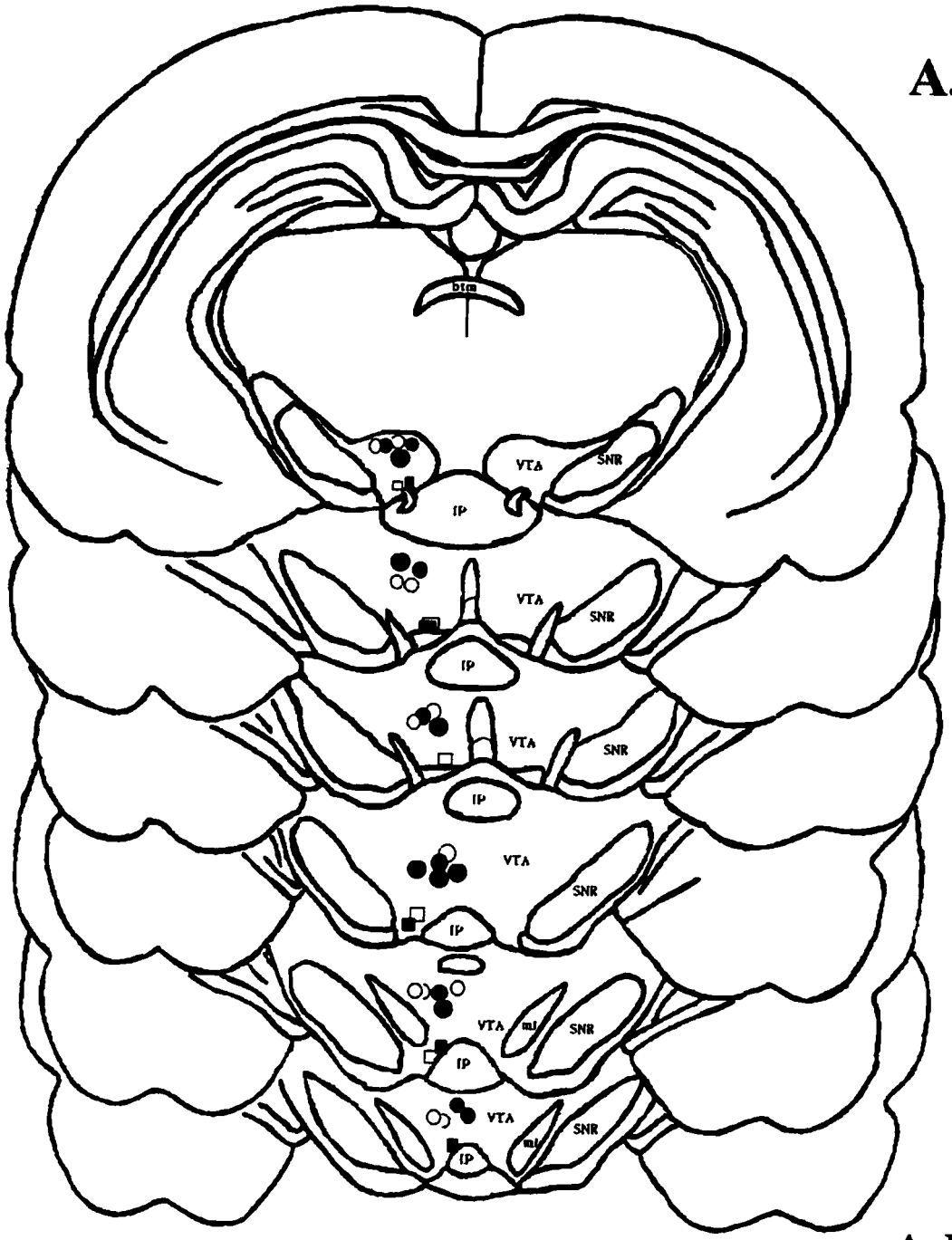

A.P. -3.0
dtm
VTA
SNR
IP
VTA
SNR
IP
VTA
SNR
IP
VTA
SNR
IP
VTA
ml
SNR
IP
VTA
ml
SNR
IP
A.P. -3.8

Figure 8.2: Baseline self-stimulation performance (± S.E.M.) among mice responding for brain stimulation from the dorsal or ventral aspects of the VTA in a descending and ascending frequency response curve. Animals were permitted to respond for brain stimulation for 3 minutes at each of the frequency parameters employed. Self-stimulation performance is expressed as a percentage of maximal baseline at 80 Hz.

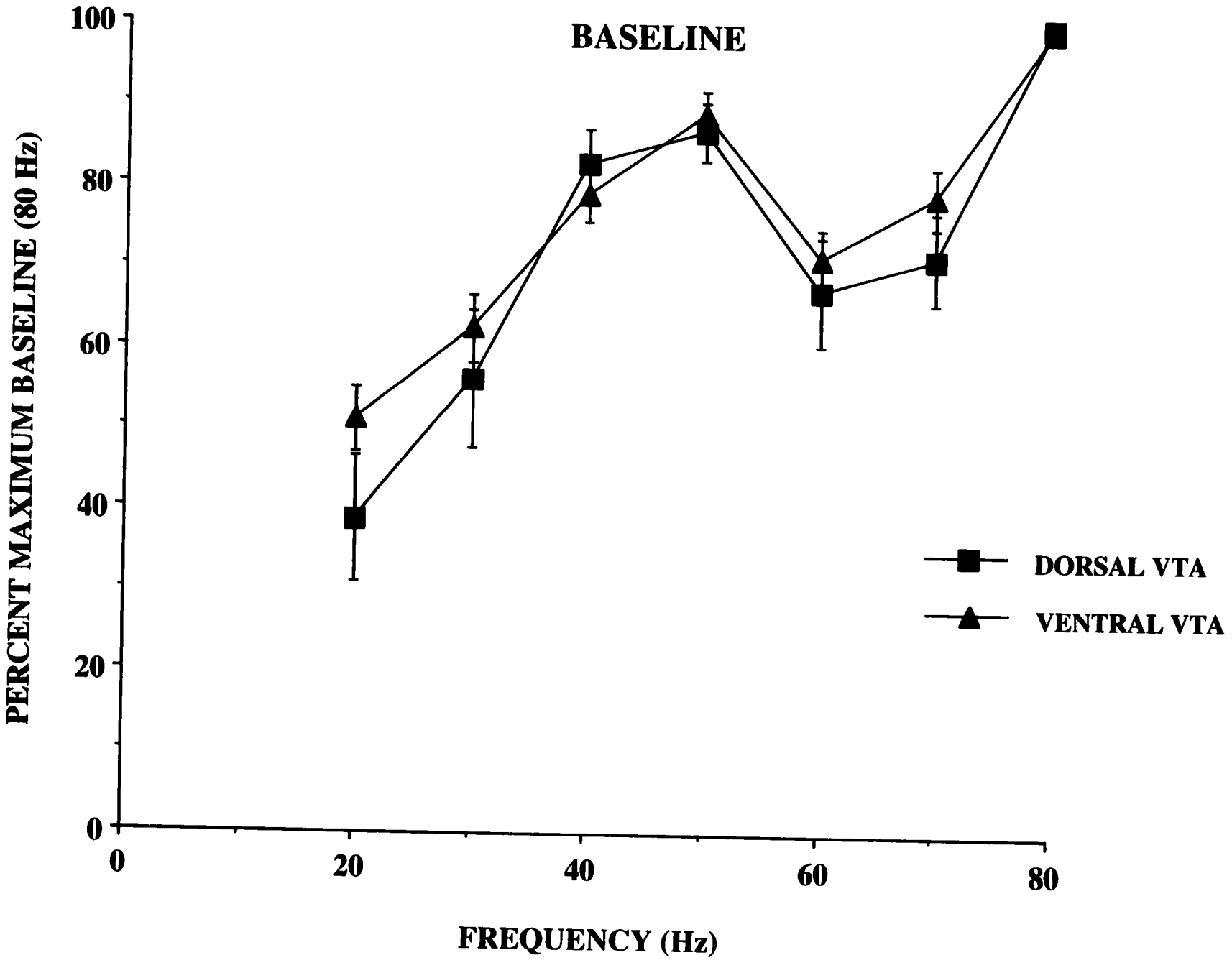
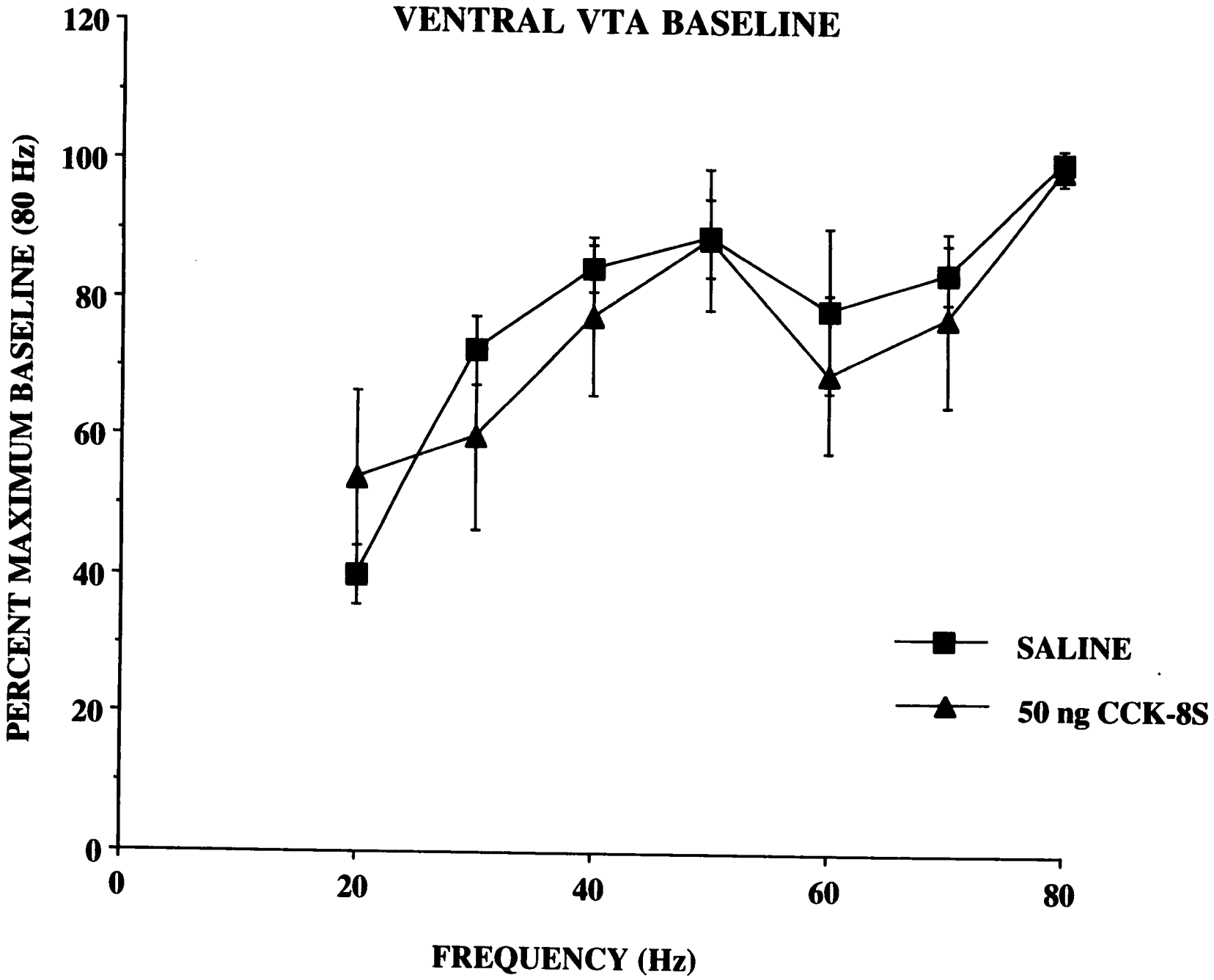

BASELINE
PERCENT MAXIMUM BASELINE (80 Hz)
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
DORSAL VTA
VENTRAL VTA

Figure 8.3: Baseline self-stimulation performance (± S.E.M.) among mice responding for brain stimulation from the ventral aspects of the VTA in a descending and ascending frequency response curve. Animals were permitted to respond for brain stimulation for 3 minutes at each of the frequency parameters employed. Self-stimulation performance is expressed as a percentage of maximal baseline at 80 Hz. Note: the saline (SAL) or CCK designation provided for the mice implanted with a stimulating electrode in the ventral VTA region reflect group assignment prior to intraventricular saline or CCK challenge.


VENTRAL VTA BASELINE
PERCENT MAXIMUM BASELINE (80 Hz)
120
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
SALINE
50 ng CCK-8S

Analysis of variance of self-stimulation scores of animals responding for brain stimulation from the ventral aspects of the VTA following intraventricular injection of saline or 50 ng CCK-8S revealed a significant effect of Frequency $F(6, 48)= 3.90$, $p<.003$) yet failed to reveal a significant difference among mice treated with either 1 µl physiological saline or 50 ng CCK-8S, $F(1, 8)= .751$, $p>.05$. Predictably, the self-stimulation performance of mice with electrodes in the ventral aspects of the VTA increased with increasing stimulation frequency. Intraventricular administration of 50 ng CCK-8S failed to induce decrements in self-stimulation performance or increase the threshold for rewarding brain stimulation (see Figure 8.4).

Insert Figure 8.4 about here

Among mice responding for brain stimulation from the dorsal aspects of the VTA (N=36), analysis of variance of baseline frequency response curves revealed a significant effect of Frequency $F(6, 168)= 134.5$, $p<.0001$) yet failed to reveal a significant difference among mice assigned to the eight treatment groups, $F(1, 28)= 1.139$, $p>.05$. Again, the baseline self-stimulation performance of mice with electrodes in the dorsal aspects of the VTA increased with increasing stimulation frequency (see Figure 8.5).

Insert Figure 8.5 about here

Figure 8.4: Self-stimulation performance (± S.E.M.) among mice responding for brain stimulation from the ventral A10 area expressed as a percentage of maximal responding of baseline at 80 Hz immediately (5 minutes) following either intraventricular saline (SAL) or CCK administration. Animals were permitted to respond for brain stimulation for 3 minutes at each of the frequency parameters employed in a descending and ascending fashion. Self-stimulation performance is expressed as a percentage of maximal baseline at 80 Hz.

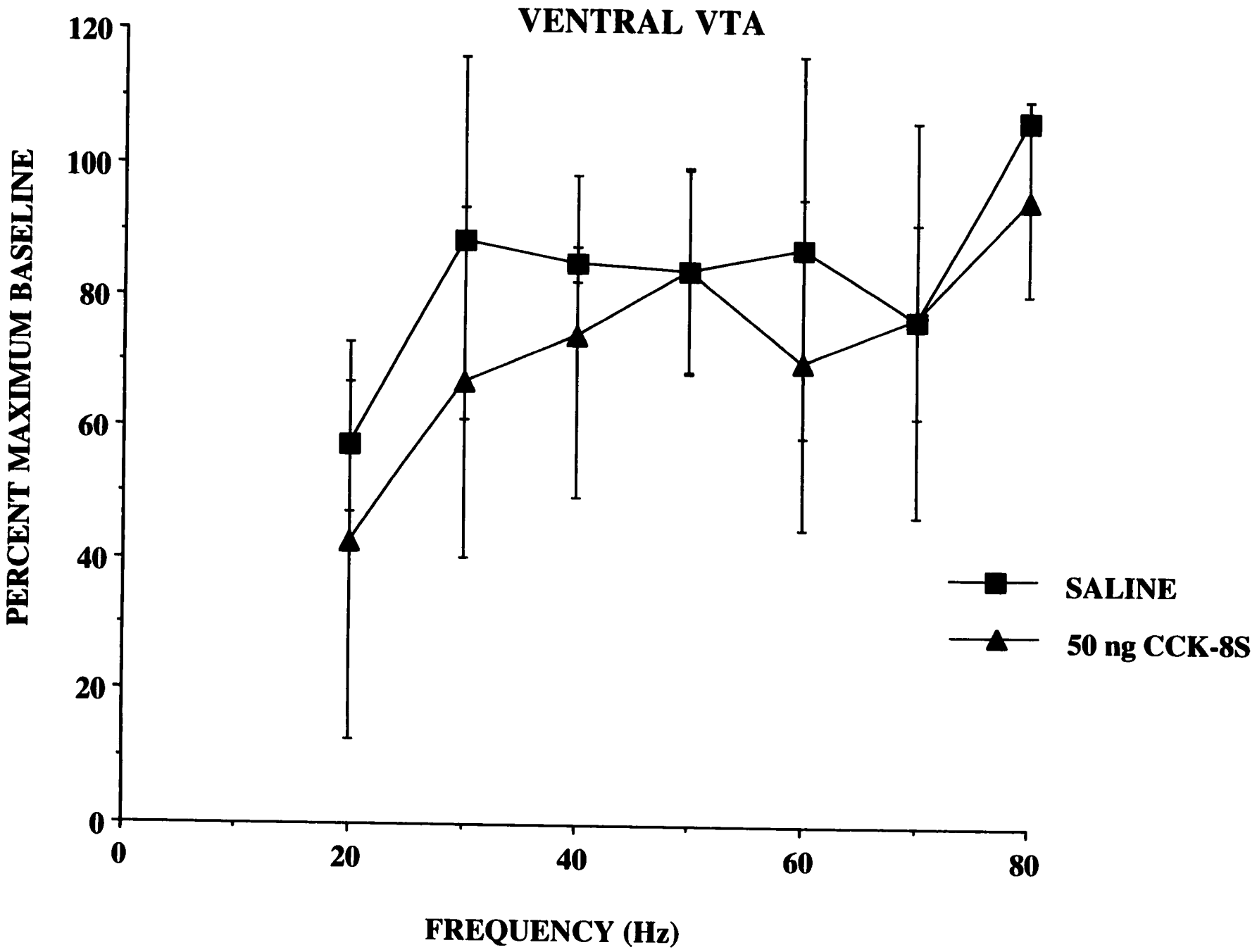
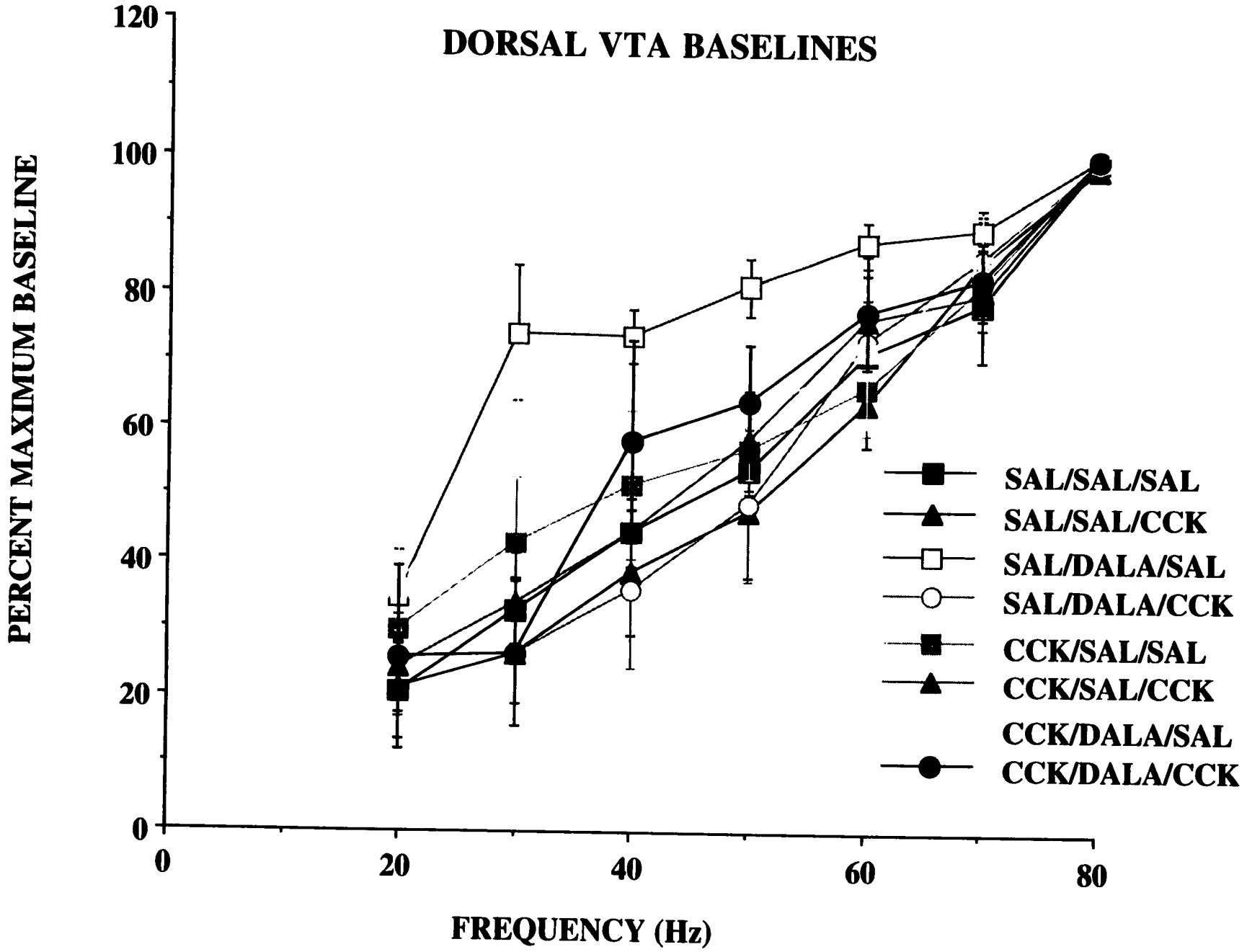

VENTRAL VTA
PERCENT MAXIMUM BASELINE
120
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
SALINE
50 ng CCK-8S

Figure 8.5: Baseline self-stimulation performance (± S.E.M.) among mice responding for brain stimulation from the dorsal aspects of the VTA in a descending and ascending frequency response curve. Animals were permitted to respond for brain stimulation for 3 minutes at each of the frequency parameters employed. Self-stimulation performance is expressed as a percentage of maximal baseline at 80 Hz. Note: the saline (SAL) or CCK designation provided for the mice implanted with a stimulating electrode in the dorsal VTA region reflect group assignment prior to intraventricular saline or CCK challenge.


DORSAL VTA BASELINES
PERCENT MAXIMUM BASELINE
120
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
SAL/SAL/SAL
SAL/SAL/CCK
SAL/DALA/SAL
SAL/DALA/CCK
CCK/SAL/SAL
CCK/SAL/CCK
CCK/DALA/SAL
CCK/DALA/CCK

Analysis of variance of self-stimulation scores following intraventricular drug administrations among animals with electrodes in the dorsal aspects of the VTA revealed a significant effect of Drug 1 $F(1, 28)= 6.852$, $p<.01$, Drug 2 $F(1, 28)= 7.072$, $p<.01$, Test Session $F(8, 224)= 13.112$, $p<.0001$), Test Session x Drug 1 $F(8, 224)= 7.731$, $p<.0001$, Test Session x Drug 2 $F(8, 224)= 1.986$, $p<.05$, Frequency $F(6, 168)= 61.905$, $p<.0001$), Frequency x Drug 1 $F(6, 168)= 5.263$, $p<.0001$, Frequency x Drug 2 $F(6, 168)= 2.735$, $p<.01$, Frequency x Drug 1 x Drug 2 $F(6, 168)= 3.182$, $p<.01$, Test Session x Frequency $F(48, 1344)= 1.462$ $p<.05$, Drug 1 x Drug 2 x Drug 3 $F(1, 28)= 8.995$, $p<.01$ and Test Session x Frequency x Drug 1 x Drug 3 $F(48, 1344)= 1.566$ $p<.01$. Analysis of variance of frequency reward thresholds among animals with electrodes in the dorsal VTA following intraventricular drug treatments revealed a significant effect of Drug 1 $F(1, 28)= 14.09$, $p<.001$, Test Session $F(8, 224)= 11.68$, $p<.0001$, Test Session x Drug 1 $F(8, 224)= 4.93$, $p<.0001$, Test Session x Drug 2 $F(8, 224)= 2.184$, $p<.05$, Test Session x Drug 1 x Drug 2 $F(8, 224)= 2.077$, $p<.05$, and Drug 1 x Drug 2 x Drug 3 $F(1, 28)= 5.45$, $p<.05$. Fisher's Least Significant Difference (LSD) multiple comparisons of the simple main effects associated with the Day x Drug 1 interaction revealed that 50 ng CCK-8S depressed self-stimulation scores across all frequencies and increased the frequency reward threshold relevant to saline treated mice in the immediate post-drug interval (see Figures 8.6 and 8.7).

Insert Figures 8.6 and 8.7 about here

Figure 8.6: Self-stimulation performance (± S.E.M.) among mice responding for brain stimulation from the dorsal A10 area expressed as a percentage of maximal responding of baseline at 80 Hz immediately (5 minutes) following either intraventricular saline (SAL) or 50 ng CCK-8S administration. Animals were permitted to respond for brain stimulation for 3 minutes at each of the frequency parameters employed in a descending and ascending fashion. Self-stimulation performance is expressed as a percentage of maximal baseline at 80 Hz.

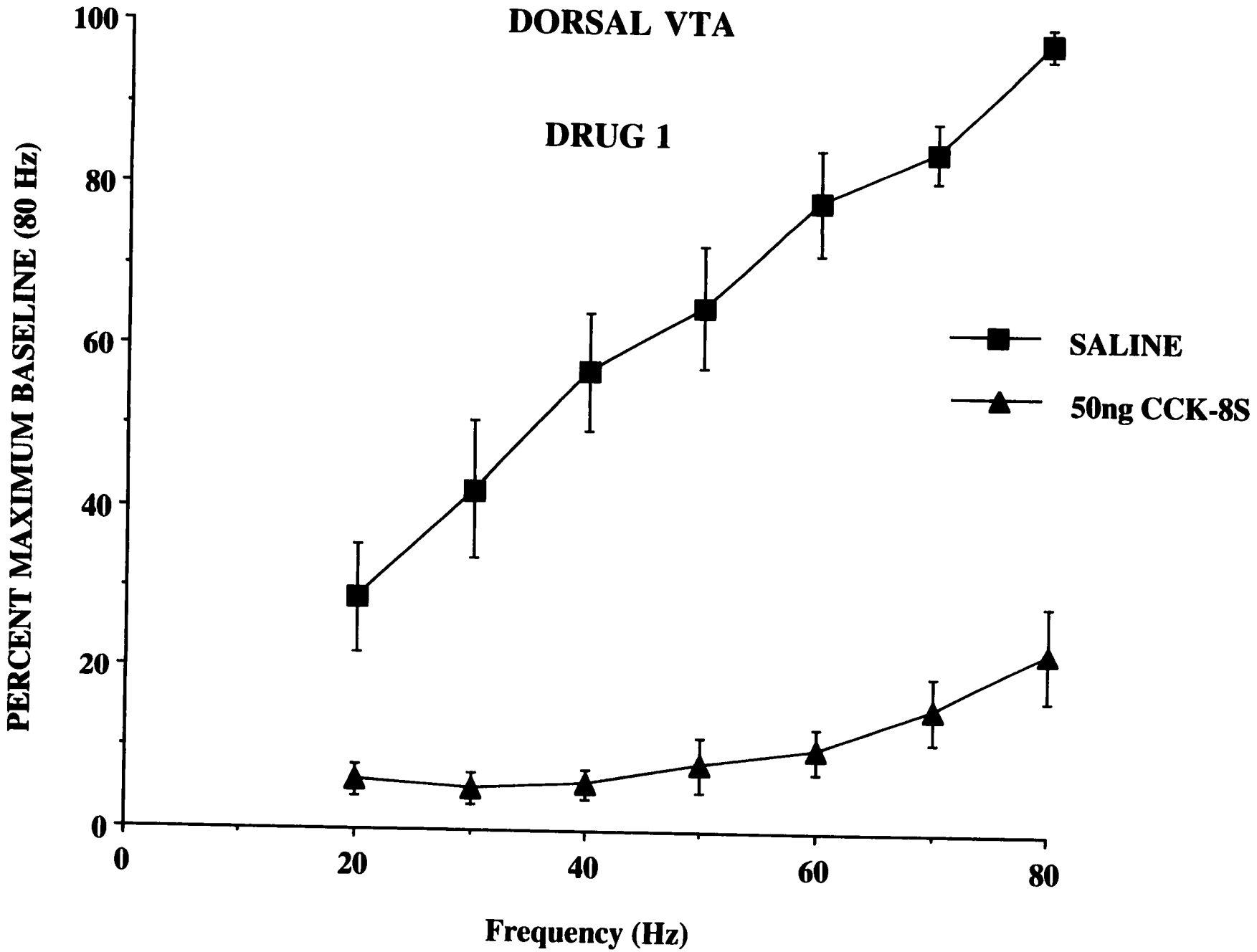
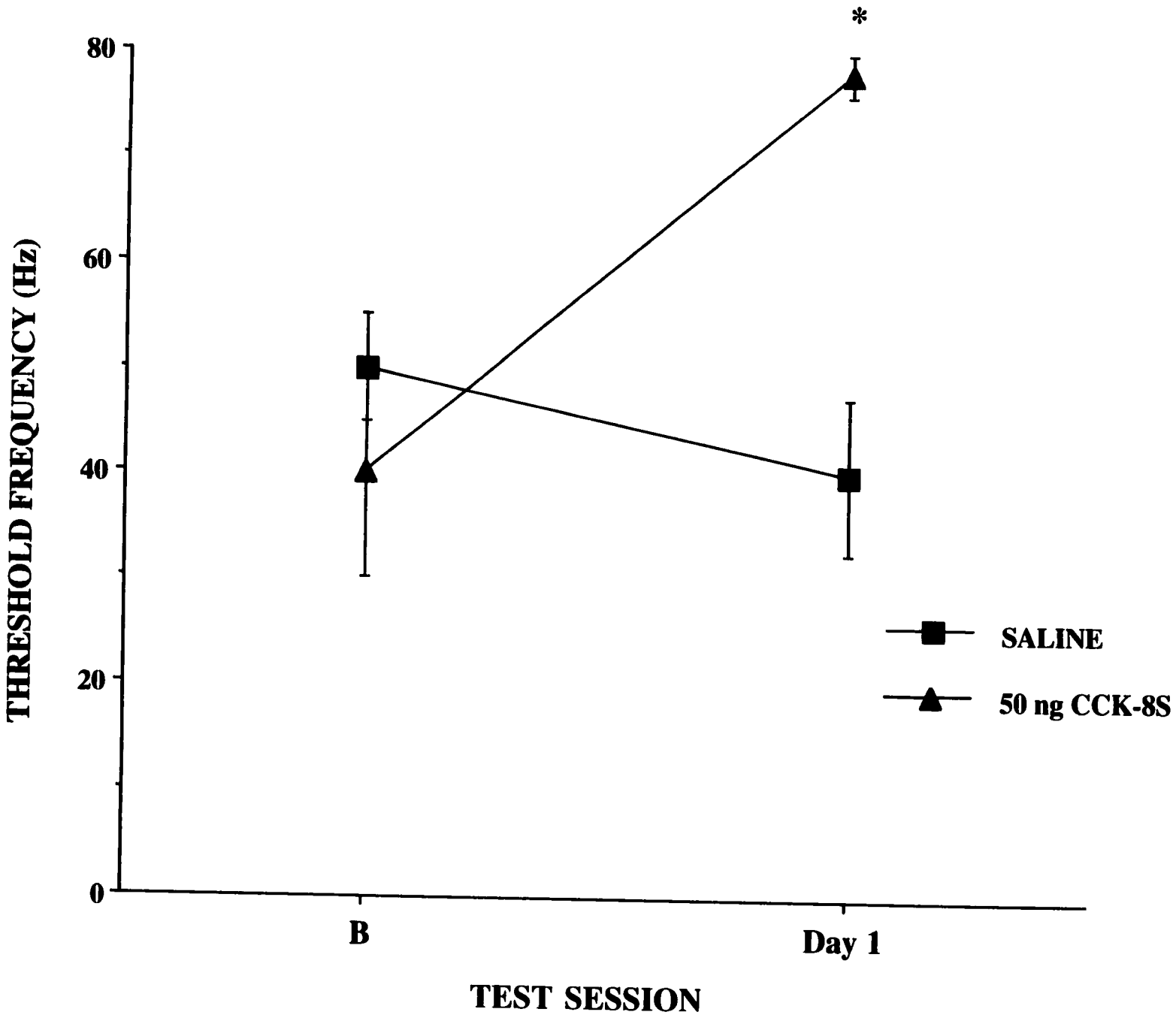

DORSAL VTA
DRUG 1
PERCENT MAXIMUM BASELINE (80 Hz)
100
80
60
40
20
0
0
20
40
60
80
Frequency (Hz)
SALINE
50ng CCK-8S

Figure 8.7: Self-stimulation frequency thresholds (± S.E.M.) among mice responding for brain stimulation from the dorsal aspects of the VTA following intraventricular administration of Drug 1: saline (SAL) or CCK-8S (50 ng in 1 µl). Note: Baseline (B) self-stimulation performance was not included in the statistical analysis of these data. The * depicts a statically significant ($p<.05$) increase in reward threshold immediately following 50 ng intraventricular CCK-8S administration.

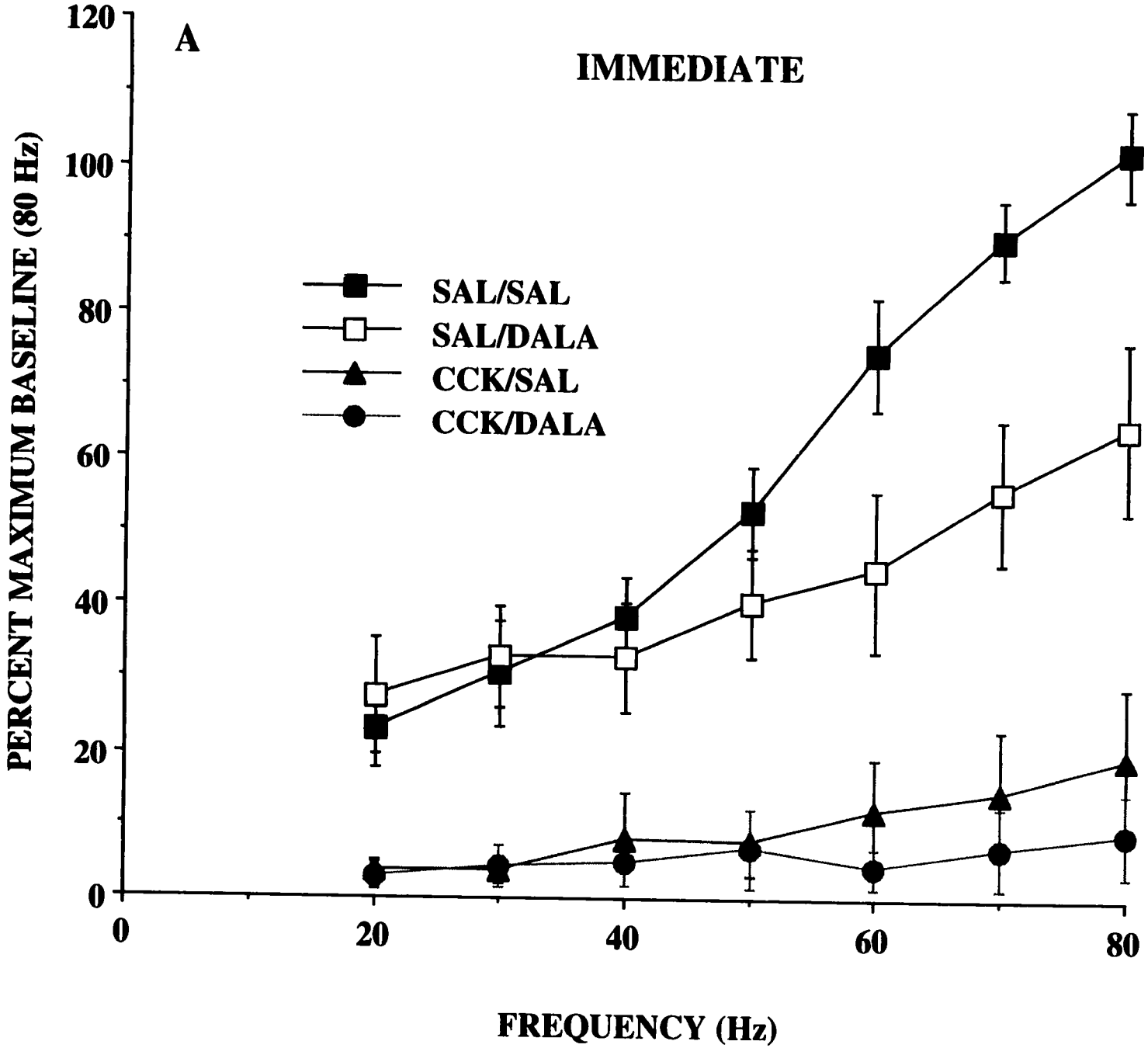

*
80
60
40
20
0
THRESHOLD FREQUENCY (Hz)
B
Day 1
TEST SESSION
SALINE
50 ng CCK-8S

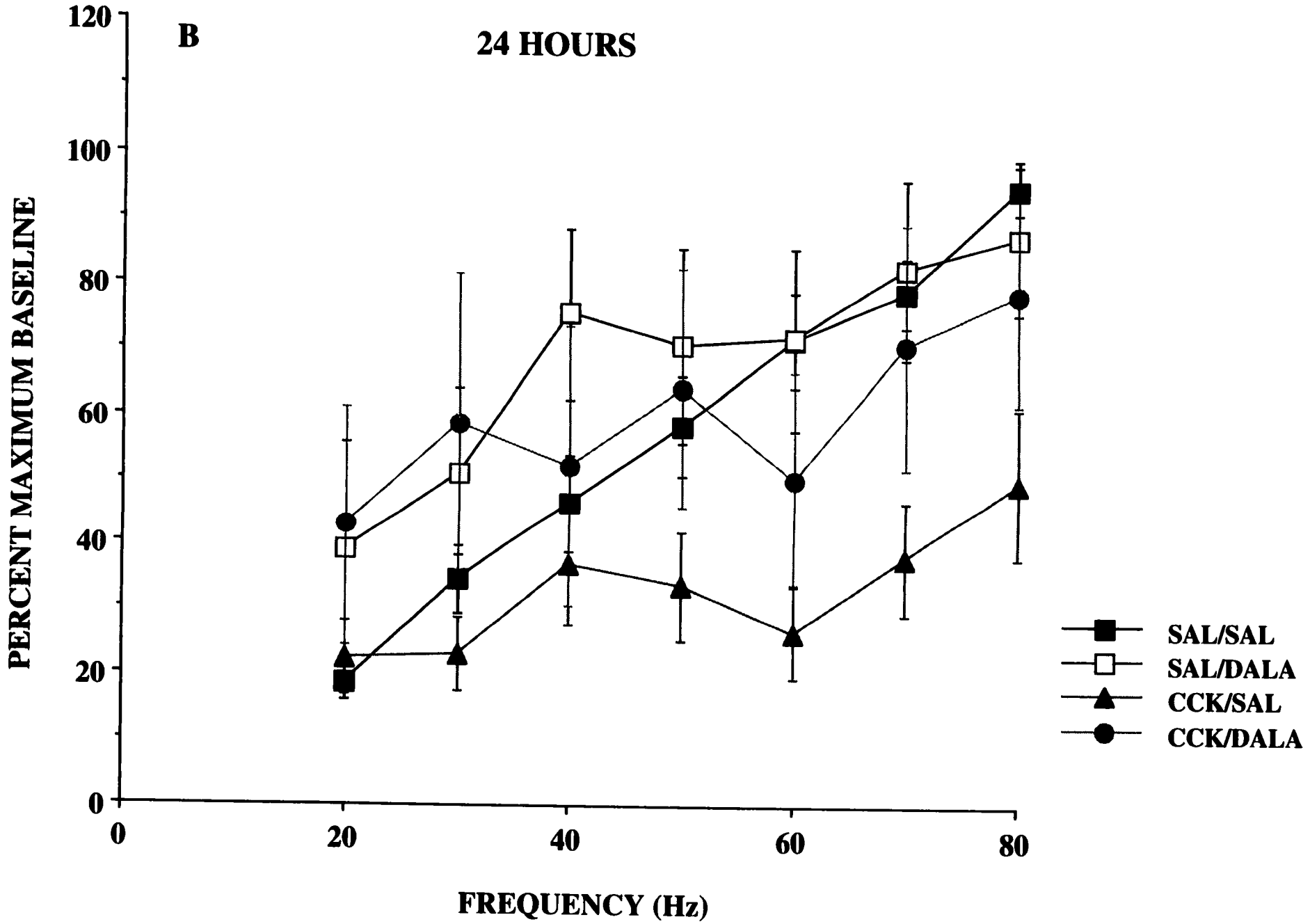
Fisher's Least Significant Difference (LSD) multiple comparisons of the simple main effects associated with the Test Session x Drug 2 interaction revealed that among CCK-8S treated mice neither SAL nor DALA administration restored brain stimulation performance or reward thresholds relative to SAL/SAL treated mice. SAL/DALA treated mice exhibited reduced self-stimulation performance at higher frequencies and elevated reward thresholds relevant to SAL/SAL treated mice during the immediate test session. CCK/SAL treated mice exhibited reduced self-stimulation performance across all frequencies and elevated reward thresholds relative to SAL/SAL, SAL/DAL and CCK/DAL mice 24 hours post-drug injections. No differences were observed among drug treatment conditions 48 or 168 hours post injection (see Figures 8.8 and 8.9).

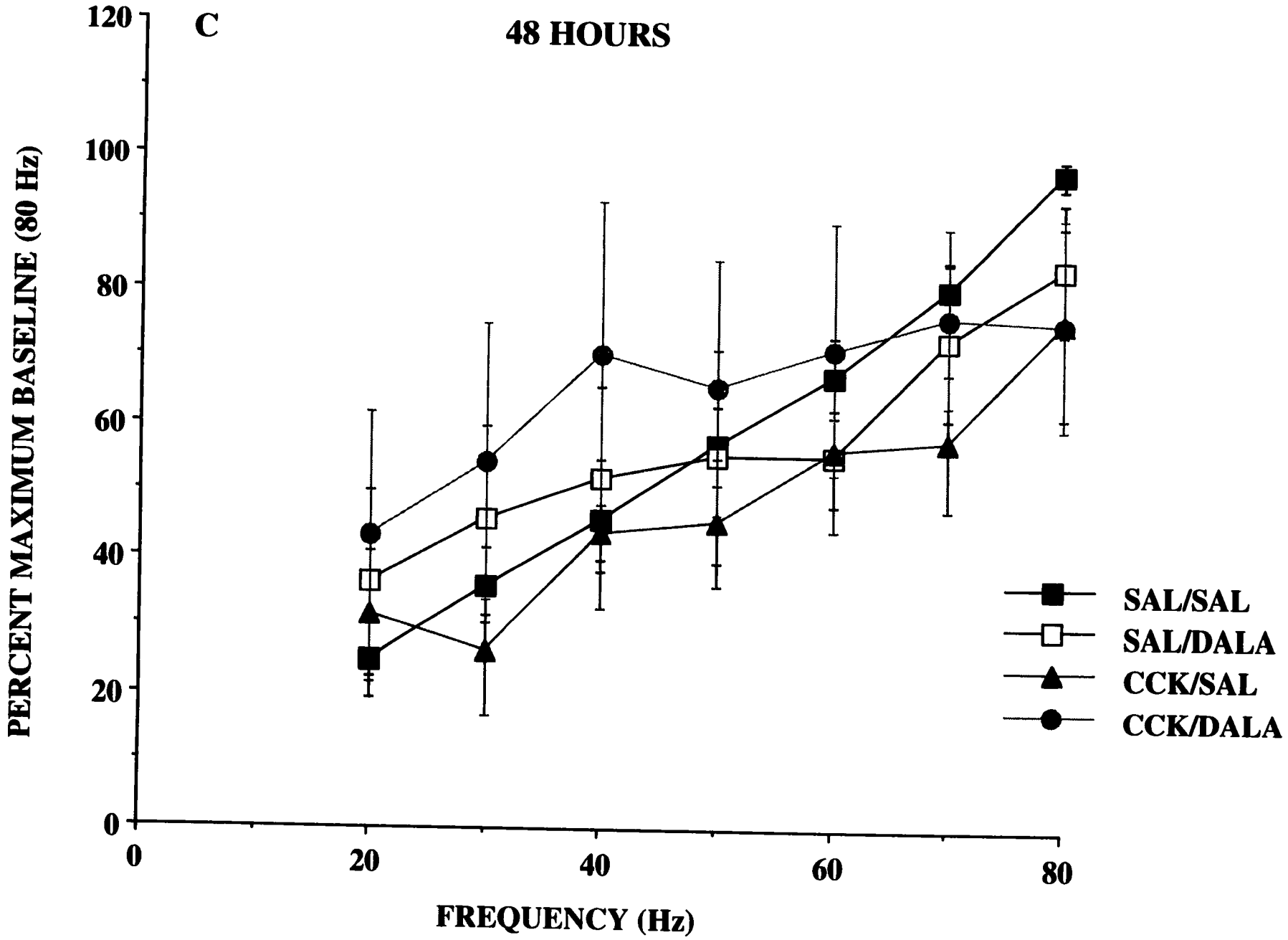
Insert Figures 8.8 and 8.9 about here

Fisher's LSD multiple comparisons of the simple simple main effects associated with the Drug 1 x Drug 2 x Drug 3 interaction revealed that CCK/SAL/CCK treated mice exhibited reduced self-stimulation scores across all frequencies and an elevated reward threshold frequency, while SAL/DALA/SAL treated mice exhibited a reduced reward threshold frequency, relative to the other 7 treatment conditions on the immediate drug re-exposure test session (Day 18). Among SALINE-treated mice there were no differences among SAL/SAL/SAL; SAL/SAL/CCK or SAL/DALA/CCK groups. Among CCK groups CCK/DALA/CCK treated mice exhibited elevated self-stimulation scores and a reduced reward threshold frequency relative to CCK/DALA/SAL and CCK/SAL/SAL treated mice. There were no differences between SAL/SAL/SAL treated mice compared to CCK/SAL/SAL

Figure 8.8: Self-stimulation performance (± S.E.M.) among mice responding for brain stimulation from the dorsal A10 area expressed as a percentage of maximal responding of baseline at 80 Hz immediately (5 minutes) (A), 24 hours (B), 48 hours (C) or 168 hours (D) following either intraventricular saline (SAL) or DALA administration. Animals were permitted to respond for brain stimulation for 3 minutes at each of the frequency parameters employed in a descending and ascending fashion. Self-stimulation performance is expressed as a percentage of maximal baseline at 80 Hz.


A
IMMEDIATE
PERCENT MAXIMUM BASELINE (80 Hz)
120
100
80
60
40
20
0
SAL/SAL
SAL/DALA
CCK/SAL
CCK/DALA
0
20
40
60
80
FREQUENCY (Hz)


B
24 HOURS
120
100
80
60
40
20
0
PERCENT MAXIMUM BASELINE
0
20
40
60
80
FREQUENCY (Hz)
SAL/SAL
SAL/DALA
CCK/SAL
CCK/DALA


C
48 HOURS
PERCENT MAXIMUM BASELINE (80 Hz)
120
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
SAL/SAL
SAL/DALA
CCK/SAL
CCK/DALA

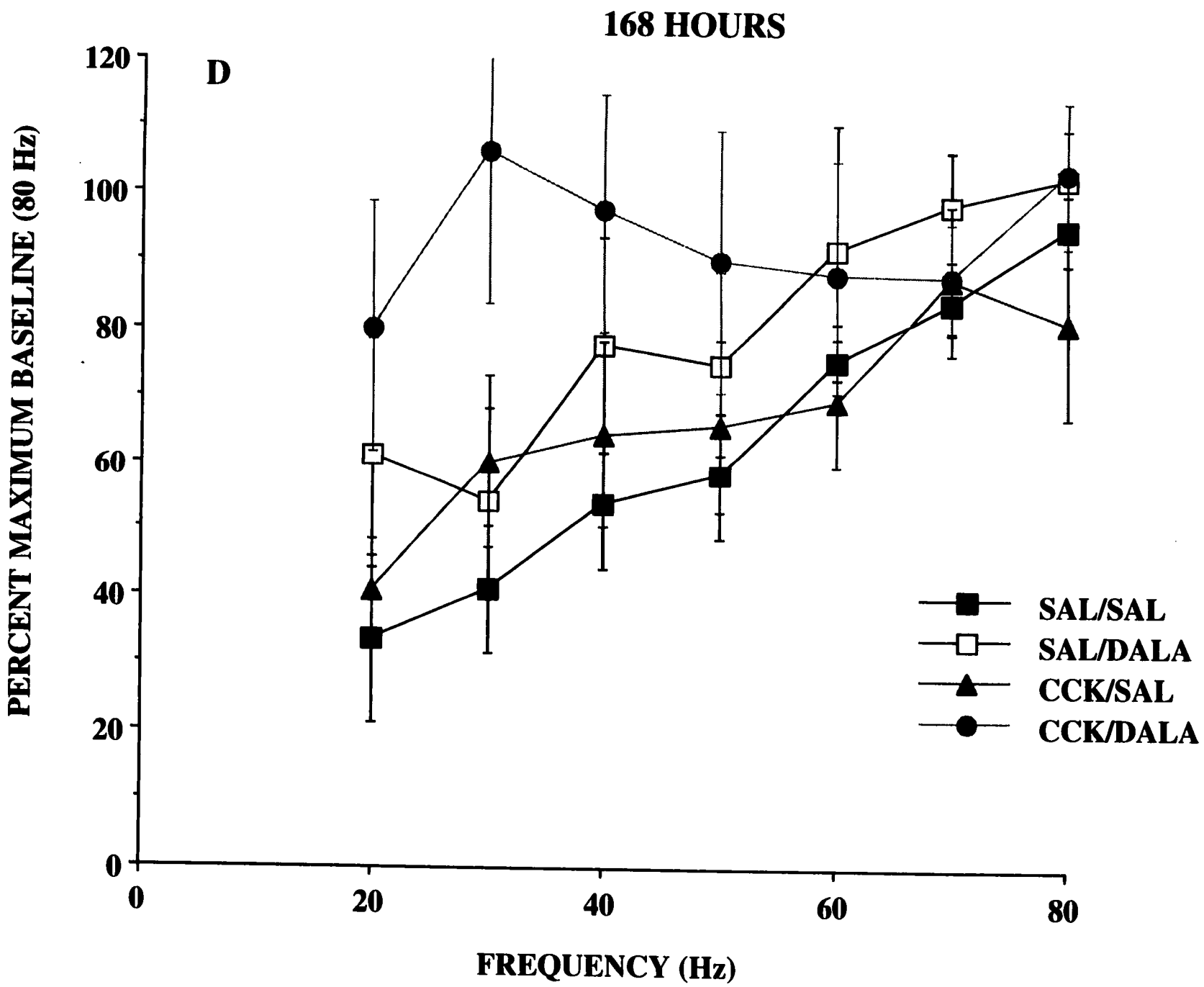
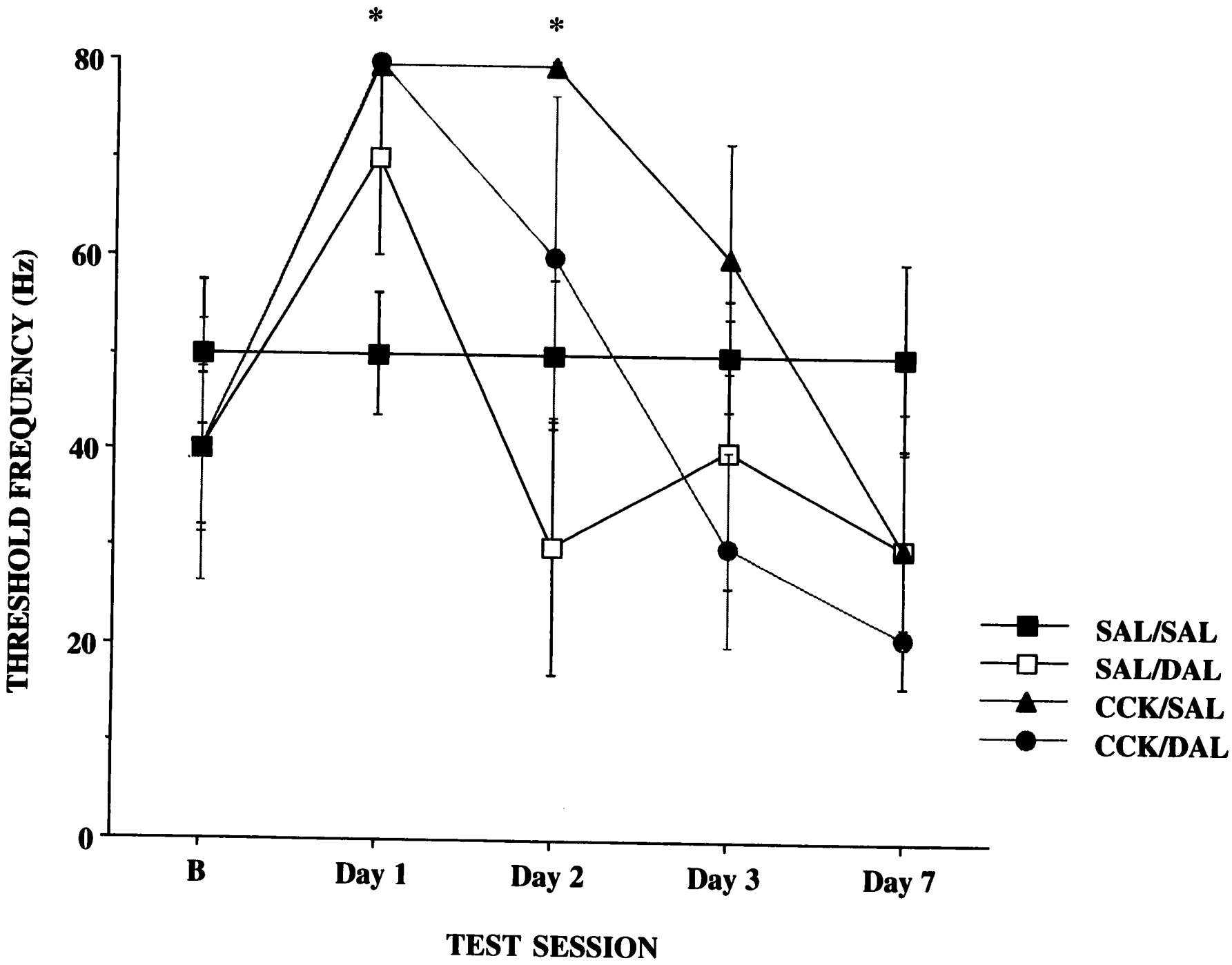

168 HOURS
D
120
100
80
60
40
20
0
0
20
40
60
80
PERCENT MAXIMUM BASELINE (80 Hz)
FREQUENCY (Hz)
SAL/SAL
SAL/DALA
CCK/SAL
CCK/DALA

Figure 8.9: Self-stimulation frequency thresholds (± S.E.M.) among mice responding for brain stimulation from the dorsal aspects of the VTA following intraventricular administration of Drug 2 saline (SAL) or DALA (1 μg in 1 μl) in mice previously administered Drug 1: SAL or 50 ng CCK-8S. Note: Baseline (B) self-stimulation performance was not included in the statistical analysis of these data. The * depicts a statically significant ($p<.05$) increase in reward threshold immediately and 24 hours following 50 ng intraventricular CCK-8S and saline (CCK/SAL) administration.


*
*
80
60
40
20
0
THRESHOLD FREQUENCY (Hz)
B
Day 1
Day 2
Day 3
Day 7
TEST SESSION
SAL/SAL
SAL/DAL
CCK/SAL
CCK/DAL

or SAL/SAL/CCK treated mice immediately following drug re-exposure (Day 18) (see Figures 8.10 and 8.11A).

Insert Figures 8.10 and 8.11 about here

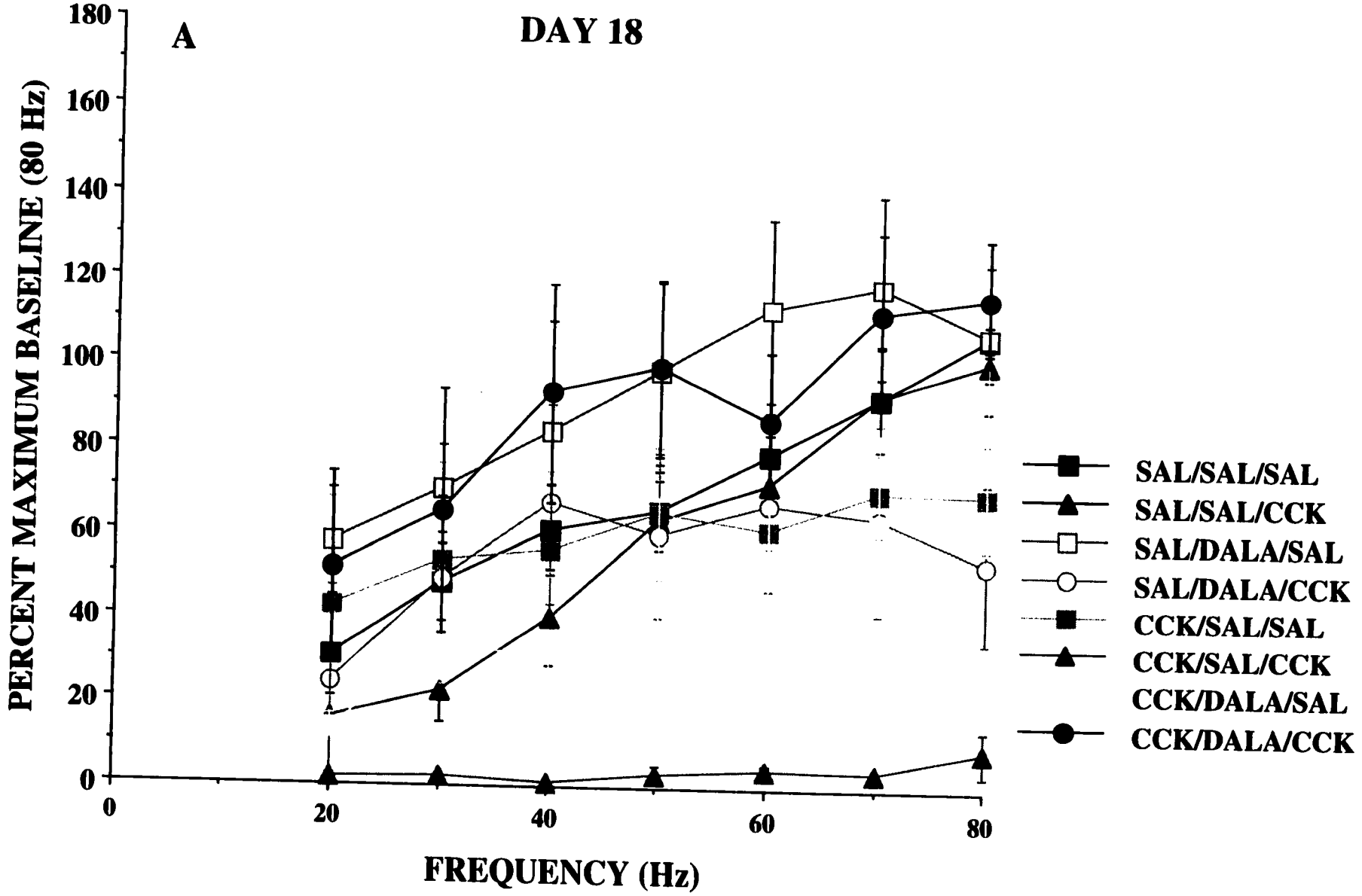
CCK/SAL/CCK treated mice exhibited reduced self-stimulation performance scores and an increased reward frequency relative to SAL/SAL/SAL and CCK/DALA/CCK treated mice 24 hours (Day 19) post-drug injection. CCK/DALA/CCK treated mice exhibited a reduced reward frequency relative to the 7 other treatment groups 24 hours (Day 19) post-drug injection (see Figures 8.10 and 8.11B). CCK/SAL/CCK treated mice exhibited reduced self-stimulation performance scores across all frequencies and increased reward threshold relative to CCK/DALA/CCK treated mice 48 hours (Day 20) post-drug injection. CCK/DALA/CCK treated mice exhibited a reduced reward frequency threshold relative to 6 treatment groups on Day 20 except SAL/DALA/SAL treated mice (see Figures 8.10 and 8.11C). There were no significant differences among any of the 8 drug treatment conditions 168 hours (Day 24) following last drug injection (see Figures 8.10 and 8.11D).

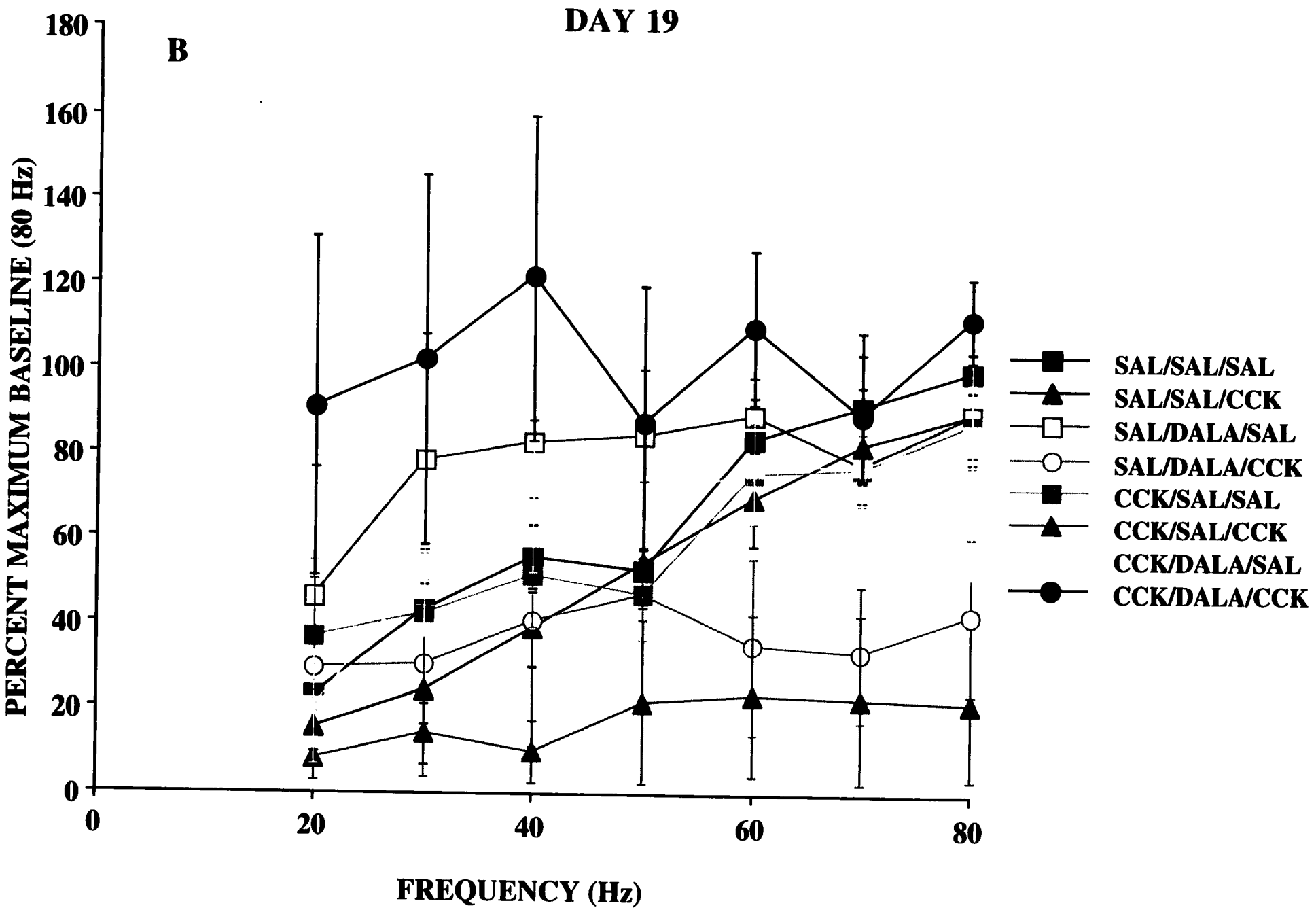
Experiment 9

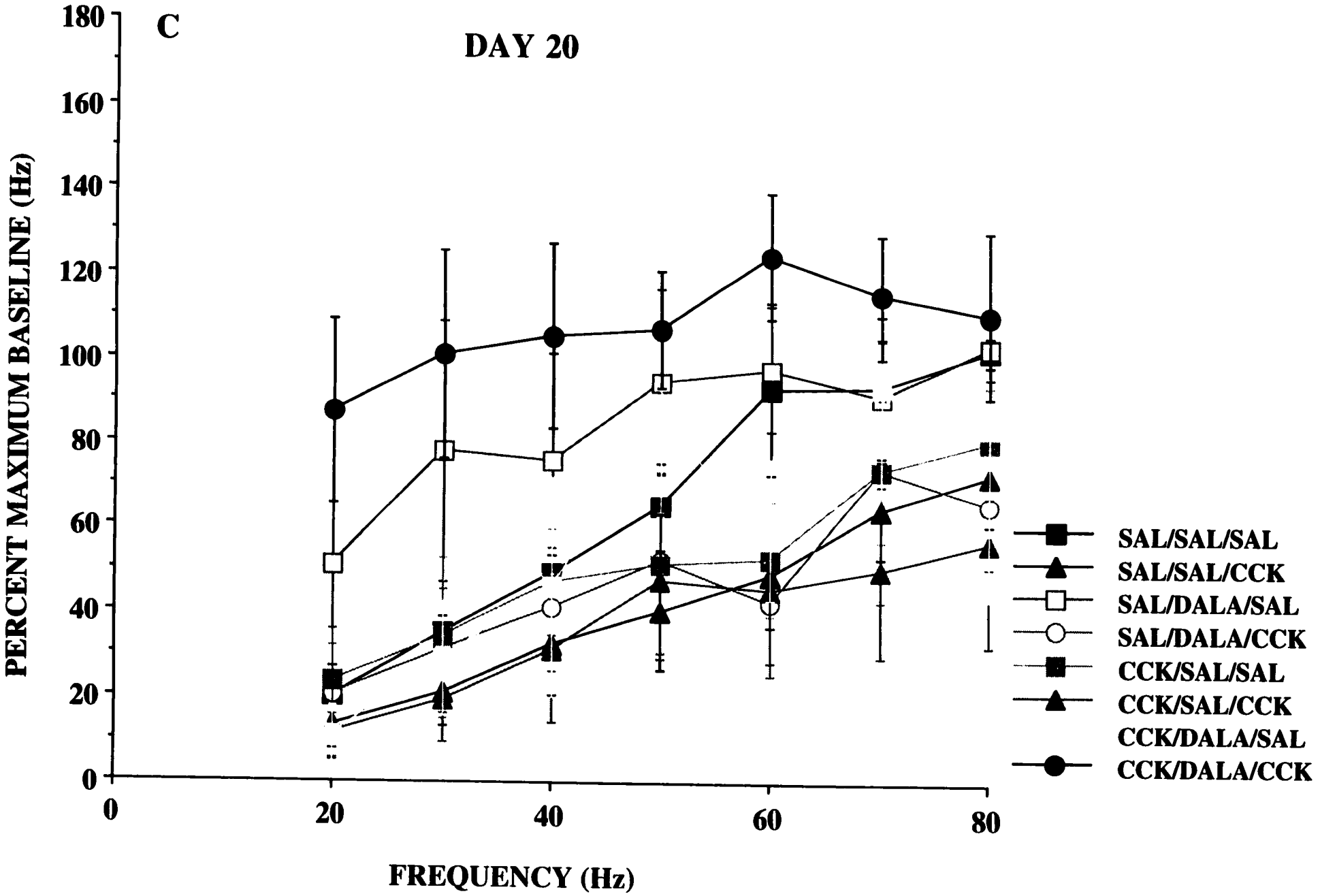
Locomotor Activity and Rearing

Activity and rearing were measured in black aluminum activity tubs (28 cm diameter x 32.5 cm high) (Carleton University Technology Center). Horizontal activity was recorded by interruption of photobeams mounted 0.5 cm above the floor of the tub. Rearing was recorded by a separate series of photobeams positioned 7 cm above the tub floor. A MacIntosh

Figure 8. 10: Self-stimulation performance (± S.E.M.) of mice responding for brain stimulation from the dorsal A10 area expressed as a percentage of maximal responding of baseline at 80 Hz immediately, Day 18 (5 minutes) (A), 24 hours, Day 19 (B), 48 hours, Day 20 (C) or 168 hours, Day 24 (D) following reexposure to either intraventricular saline (SAL) or 5 ng CCK-8S administration. Animals were permitted to respond for brain stimulation for 3 minutes at each of the frequency parameters employed in a descending and ascending fashion. Self-stimulation performance is expressed as a percentage of maximal baseline at 80 Hz.


A
DAY 18
PERCENT MAXIMUM BASELINE (80 Hz)
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
SAL/SAL/SAL
SAL/SAL/CCK
SAL/DALA/SAL
SAL/DALA/CCK
CCK/SAL/SAL
CCK/SAL/CCK
CCK/DALA/SAL
CCK/DALA/CCK


DAY 19
B
PERCENT MAXIMUM BASELINE (80 Hz)
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
SAL/SAL/SAL
SAL/SAL/CCK
SAL/DALA/SAL
SAL/DALA/CCK
CCK/SAL/SAL
CCK/SAL/CCK
CCK/DALA/SAL
CCK/DALA/CCK


C
DAY 20
PERCENT MAXIMUM BASELINE (Hz)
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
SAL/SAL/SAL
SAL/SAL/CCK
SAL/DALA/SAL
SAL/DALA/CCK
CCK/SAL/SAL
CCK/SAL/CCK
CCK/DALA/SAL
CCK/DALA/CCK

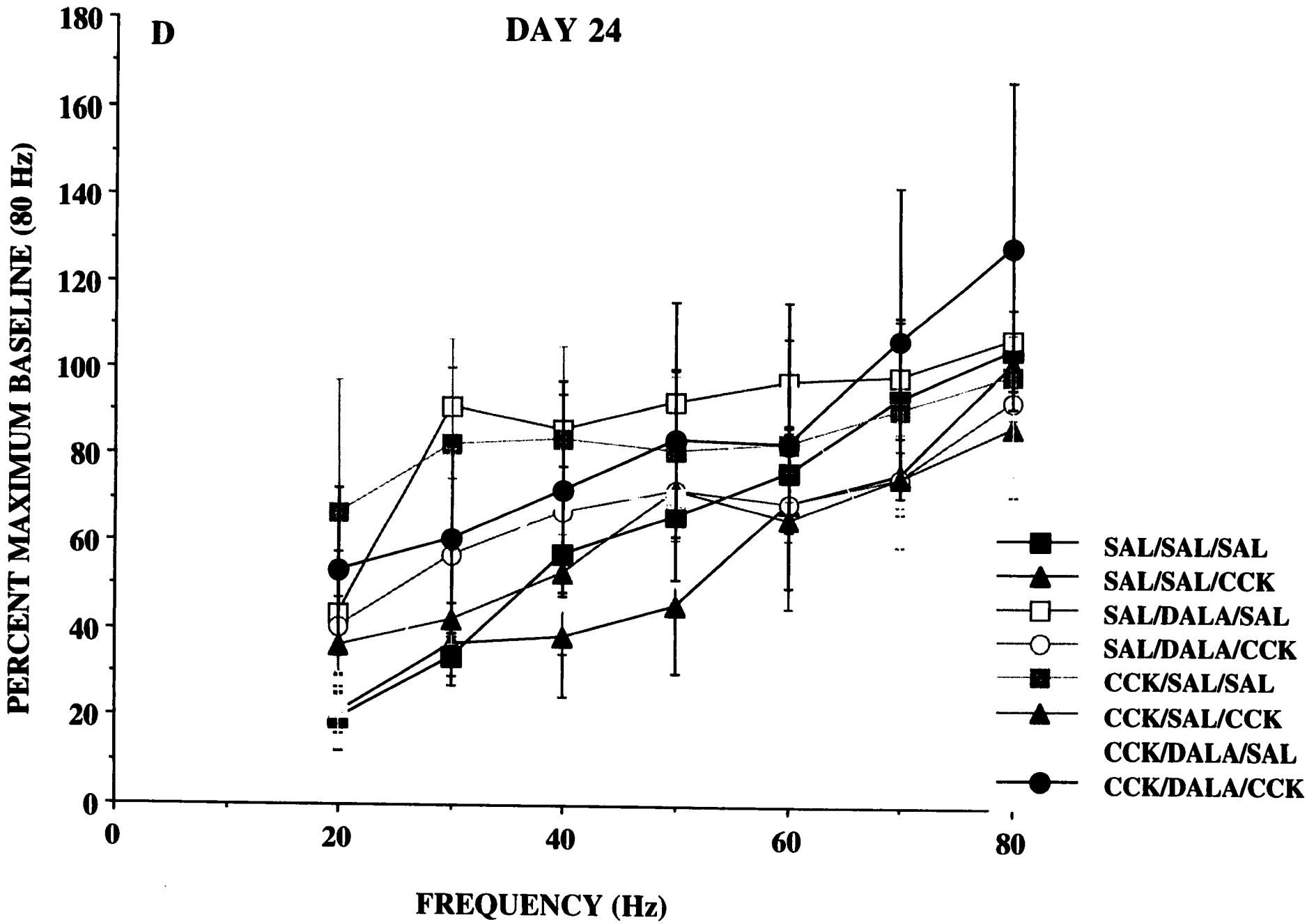
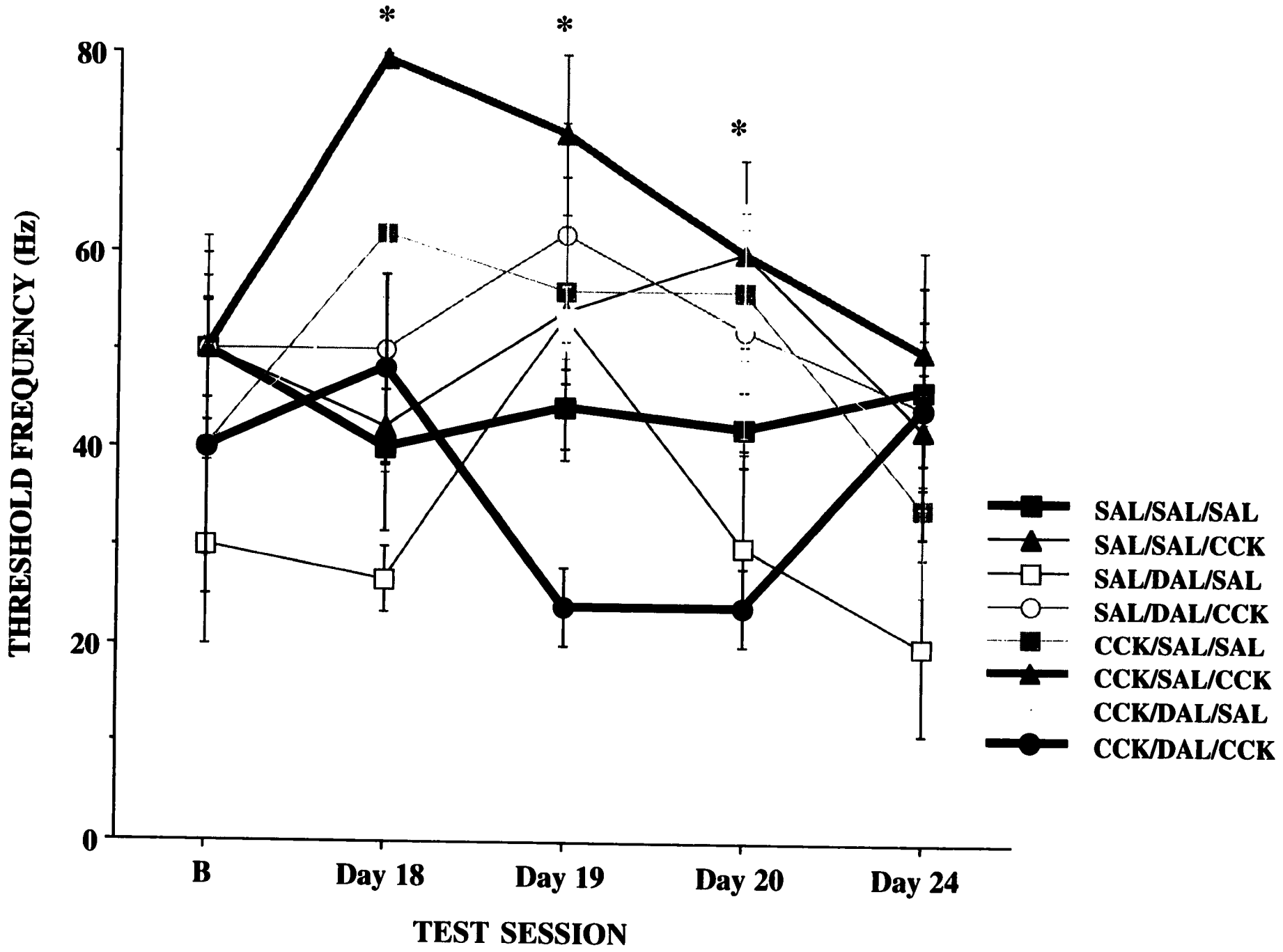

D
DAY 24
PERCENT MAXIMUM BASELINE (80 Hz)
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
FREQUENCY (Hz)
SAL/SAL/SAL
SAL/SAL/CCK
SAL/DALA/SAL
SAL/DALA/CCK
CCK/SAL/SAL
CCK/SAL/CCK
CCK/DALA/SAL
CCK/DALA/CCK

Figure 8.11: Self-stimulation frequency thresholds (± S.E.M.) among mice responding for brain stimulation from the dorsal aspects of the VTA immediately (5 minutes) (A), 24 hours (B), 48 hours (C) or 168 hours (D) following reexposure to either intraventricular saline (SAL) or 5 ng CCK-8S (Drug 3) administration. Note: Baseline (B) self-stimulation performance was not included in the statistical analysis of these data. The * depicts a statically significant ($p<.05$) difference in reward threshold immediately (Day 18), 24 hours (day 19) and 48 hours (Day 20) following reexposure to 5 ng CCK-8S or saline administration. Reexposure to 5 ng CCK-8S increased reward threshold in mice previously administered CCK/SAL and decreased reward thresholds in mice previously administered CCK/DALA relative to each other and to SAL/SAL/SAL treated mice (lines in bold).


THRESHOLD FREQUENCY (Hz)
80
60
40
20
0
*
*
*
B
Day 18
Day 19
Day 20
Day 24
TEST SESSION
SAL/SAL/SAL
SAL/SAL/CCK
SAL/DAL/SAL
SAL/DAL/CCK
CCK/SAL/SAL
CCK/SAL/CCK
CCK/DAL/SAL
CCK/DAL/CCK

computer using a Tub Monitor program (Schnabel Electronics, Saskatoon, Saskatchewan) recorded all behavioural indices.

Following postoperative recovery, all mice were introduced to the locomotor tubs in order to establish baseline levels of activity. Measures of horizontal locomotor activity and rearing were recorded at three-minute intervals over two 45-minute test sessions (Baseline A and Baseline B). Animals were tested for 45 minutes (Baseline A) and then removed from the tub and handled for approximately 2 minutes in a manner which simulated handling during intraventricular drug administration. Mice were then reintroduced into the tub and tested for a subsequent 45 minutes (Baseline B). This procedure was followed for three days and the behavioural data derived from Days 2 and 3 were used to calculate total mean activity levels (Baseline A and Baseline B) across the two measures of behaviour. Two separate baselines (Baseline A and Baseline B) were established as mice failed to exhibit stable locomotor and rearing responses (< 10% variability) over the 2 separate 45 minute tests. In fact, mice exhibited up to a 50% reduction of rearing and locomotor responses during the second 45 minute test session relative to the first 45 minute test session.

On Day 1 of behavioural testing, mice received either 50 ng CCK-8S (n=16) or saline (n=16). Intraventricular injections were accomplished as previously described in Experiment 8. Following injection, the stylette was reinserted and the animal was returned to the locomotor tub and tested for 45 minutes. Mice were then removed from the tubs and challenged with 1.0 μg of DALA (n=16) or physiological saline (n=16) and reassessed for locomotor and rearing activity. Mice were retested 24 hours (Day 2), 48 hours (Day 3) and 168 hours (Day 7) following initial drug injections. Eighteen days following initial drug

treatment (or 11 days following 168h test) mice received either 5 ng CCK-8S (n=16) or physiological saline (n=16). Mice were assessed for locomotor performance immediately (Day 18), 24 hours (Day 19), 48 hours (Day 20) and 168 hours (Day 24) following 5 ng CCK-8S or physiological saline administration (n=4 per 8 treatment groups). Drug injections occurred on Day 1 (50 ng CCK-8S/Saline, Drug 1; 1μg DALA/Saline, Drug 2) and Day 18 (5 ng CCK-8S/Saline, Drug 3), only.

Data Analysis

Locomotor activity and rearing scores on Day 1 in response to either 50 ng CCK-8S or physiological saline (Drug 1) were converted to a percentage of Baseline A where the behavioural measures in response to Drug 2 on Day 1 (1 μg DALA or saline) were converted to a percentage of Baseline B. All subsequent scores (Days 2,3,4,7,18,19,20 & 24) were converted to a percentage of Baseline A. Locomotor and rearing percent scores were subjected to ANOVA as a 2 (CCK or Saline) x 2 (DALA or Saline) x 2 (CCK or Saline) design with repeated measures over sessions (9). Separate analyses of variance were run for rearing and locomotor scores. Fisher's least significant difference (LSD) multiple comparisons were employed where appropriate and the .05 level of significance was adopted for all comparisons.

RESULTS

Locomotor Activity

Analysis of variance of locomotor activity scores among mice following drug injections revealed a significant effect of Test Session $F(8,192)=20.81$, $p<.001$, Drug 1 $F(1,24)=5.40$, $p<.05$, Drug 2 $F(1,24)=23.87$, $p<.001$, Test Session x Drug 1 $F(8,192)=3.30$,

$p<.01$, Test Session x Drug 2 $F(8, 192)=22.65$, $p<.001$, Test Session x Drug 1 x Drug 2 $F(8,192)=2.73$, $p<.01$, Test Session x Drug 1 x Drug 3 $F(8, 192) = 4.77$, $p<.001$ and Test Session x Drug 1 x Drug 2 x Drug 3 $F(8, 192)= 4.79$, $p<.001$. 50 ng CCK-8S depressed locomotor activity relative to saline treated mice in the immediate post-Drug 1 interval on Day 1 (see Figure 9.1).

Insert Figure 9.1 about here

Fisher's Least Significant Difference (LSD) multiple comparisons ($\alpha=.05$) of the simple simple main effects associated with the Test Session x Drug 1 x Drug 2 interaction revealed that administration of DALA increased locomotor activity in animals previously treated with saline and 50 ng CCK-8S. Administration of DALA increased locomotor activity in saline treated mice compared to mice treated with 50 ng CCK-8S in the immediate post-Drug 2 interval only. Administration of DALA among CCK treated mice also increased locomotor activity relative to SALINE-SALINE treated mice on Day 1 (see Figures 9.2 and 9.3).

Insert Figures 9.2 and 9.3 about here

Figure 9.1: Locomotor activity (± S.E.M.) expressed as a percentage of Baseline immediately (5 minutes) among mice following intraventricular saline (SAL) or 50 ng CCK-8S administration. Locomotor activity was assessed for 45 minutes following either SAL or CCK challenge. The * depicts a statically significant ($p<.05$) decrease in locomotor activity among mice following 50 ng intraventricular CCK-8S relative to saline administration.

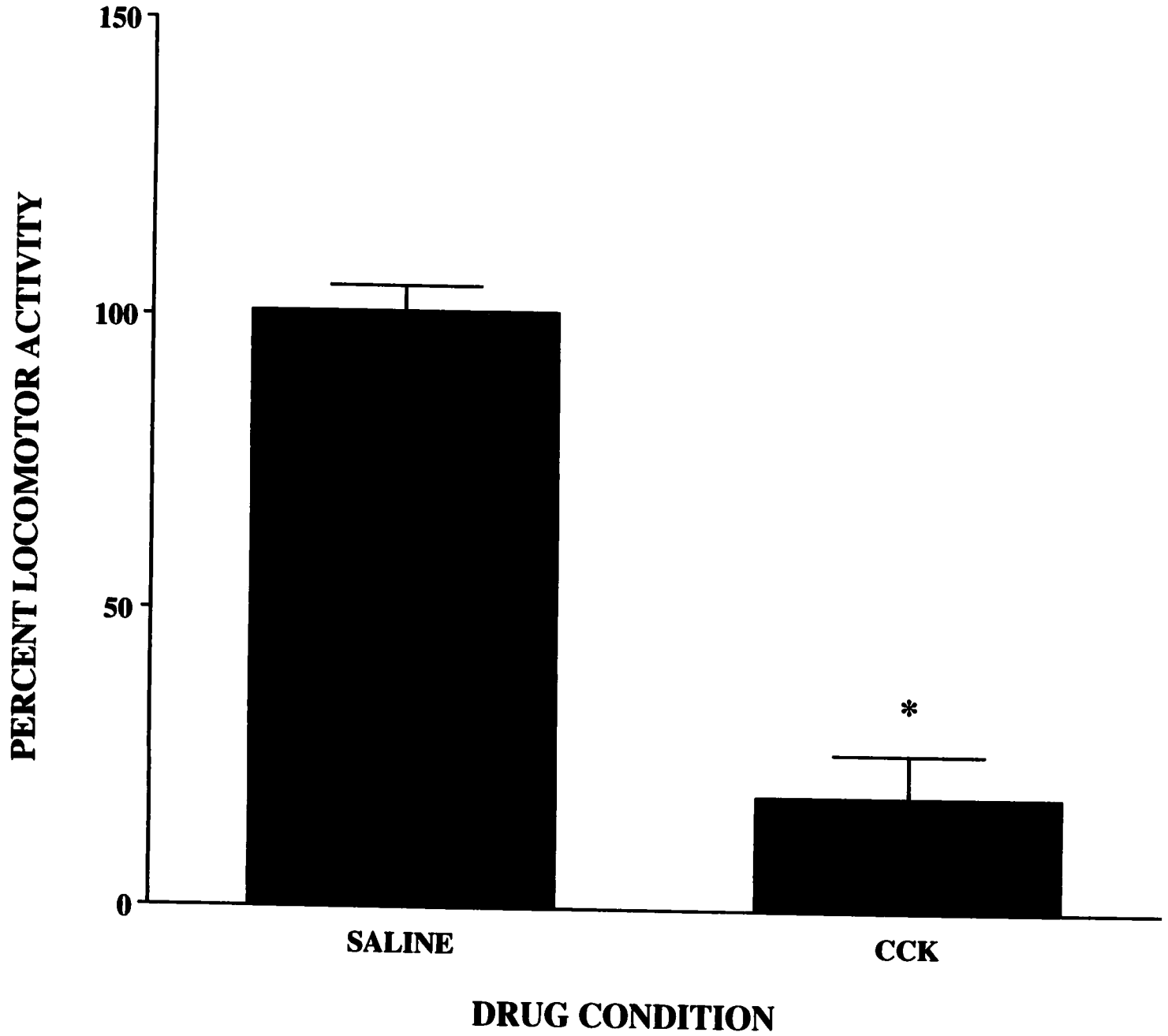

150
100
50
0
PERCENT LOCOMOTOR ACTIVITY
*
SALINE
CCK
DRUG CONDITION

Figure 9.2: Locomotor activity (± S.E.M.) expressed as a percentage of Baseline immediately (5 minutes) (A), 24 hours (B), 48 hours (C) or 168 hours (D) following SAL administration among mice previously treated with SAL or 50 ng CCK-8S. Locomotor activity was assessed for 45 minutes following SAL challenge. The * depicts a statically significant ($p<.05$) decrease in locomotor activity following 50 ng intraventricular CCK-8S/SAL relative to SAL/SAL administration at the identical test interval.

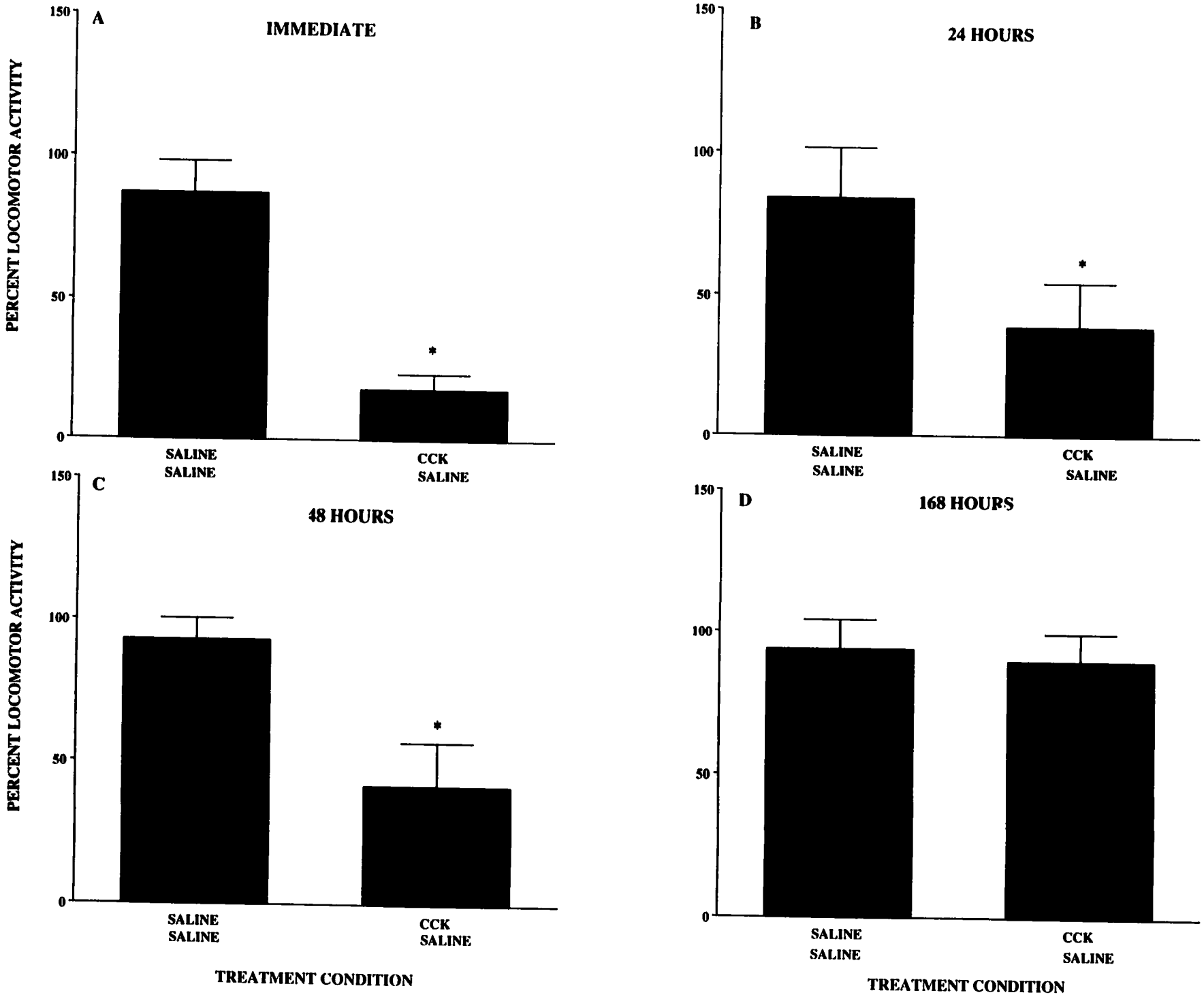
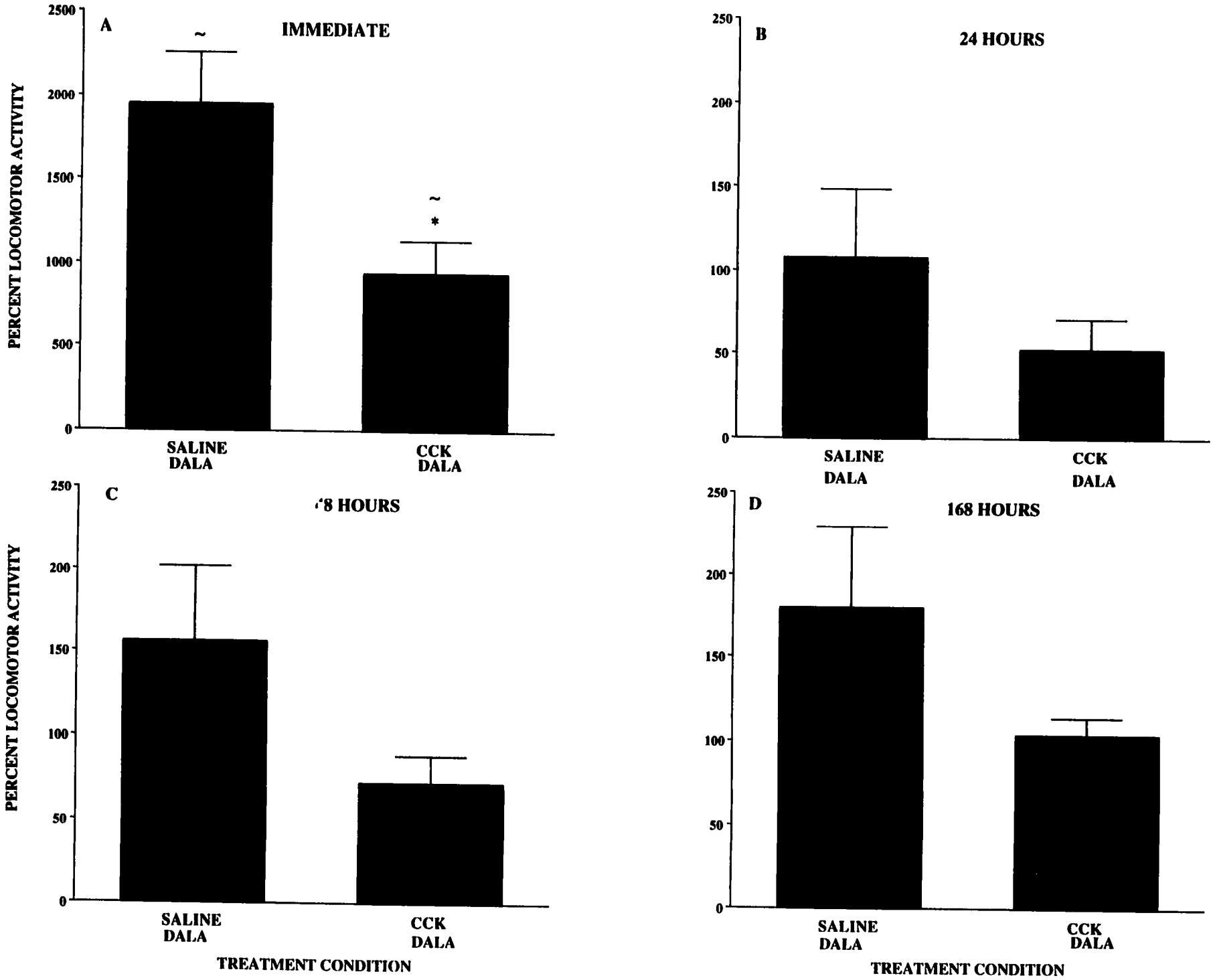

A
IMMEDIATE
B
24 HOURS
C
48 HOURS
D
168 HOURS
PERCENT LOCOMOTOR ACTIVITY
150
100
50
0
*
SALINE
SALINE
CCK
SALINE
TREATMENT CONDITION

Figure 9.3: Locomotor activity (± S.E.M.) expressed as a percentage of Baseline immediately (5 minutes) (A), 24 hours (B), 48 hours (C) or 168 hours (D) following DALA administration in mice previously treated with SAL or 50 ng CCK-8S. Locomotor activity was assessed for 45 minutes following DALA challenge. Note: * depicts a statically significant ($p<.05$) difference in locomotor activity following 50 ng intraventricular CCK-8S/DALA relative to SAL/DALA administration at the identical test interval. The ~ depicts a statically significant ($p<.05$) difference in locomotor activity following SAL/DALA or CCK/DALA relative to SAL/SAL or CCK/SAL (Figure 9.2) at the identical test interval.


A
IMMEDIATE
B
24 HOURS
C
168 HOURS
D
168 HOURS
PERCENT LOCOMOTOR ACTIVITY
2500
2000
1500
1000
500
0
250
200
150
100
50
0
~
*
SALINE
DALA
CCK
DALA
TREATMENT CONDITION

Fisher's LSD multiple comparisons of the simple simple simple main effects associated with the higher order interaction Test Session x Drug 1 x Drug 2 x Drug 3 revealed that the locomotor activity of CCK-DALA, CCK-SALINE, and SALINE-SALINE animals were comparable among animals which received 5 ng of CCK-8S or saline in the immediate post-drug interval on Day 18. Among SALINE-DALA mice, locomotor activity was depressed in animals which received 5 ng of CCK-8S compared to saline in the immediate post-drug interval. Five nanograms of CCK-8S increased locomotor activity among CCK-DALA animals in comparison to SALINE-DALA animals in the immediate post-drug interval (see Figure 9.4).

Insert Figure 9.4 about here

Rearing

Analysis of rearing scores revealed a significant effect of Test Session $F(8,192) = 14.14$, $p < .001$, Drug 1 $F(1,24) = 7.38$, $p < .05$, Drug 2 $F(1, 24) = 8.52$, $p < .001$, Test Session x Drug 2 $F(8, 192) = 16.84$, $p < .001$ and Drug 1 x Drug 2 $F(1,24) = 3.75$, $p < .06$. 50 ng CCK-8S depressed rearing relative to saline treated mice immediately following Drug 1 administration (see Figure 9.5).

Insert Figure 9.5 about here

Fisher's LSD multiple comparisons ($\alpha = .05$) of the simple main effects associated with the Drug 1 x Drug 2 interaction showed that CCK/SALINE treated mice exhibited reduced rearing

Figure 9.4: Locomotor activity (± S.E.M.) expressed as a percentage of Baseline immediately (5 minutes) (Day 18) , 24 hours (Day 19), 48 hours (Day 20) or 168 hours (Day 24) following reexposure to SAL or 5 ng CCK-8S in mice previously treated with SAL (top panel) or 50 ng CCK-8S (bottom panel). Locomotor activity was assessed for 45 minutes following SAL or CCK challenge. Note: * depicts a statically significant ($p<.05$) difference in locomotor activity comparing treatment groups in the top panel with treatment groups immediately below it in the bottom panel (i.e. SAL/DAL/CCK with CCK/DALA/CCK) following reexposure to 5 ng intraventricular CCK-8S or saline among. The ~ depicts a statically significant ($p<.05$) difference in locomotor activity among treatment groups relative to SAL/SAL/SAL treated mice.

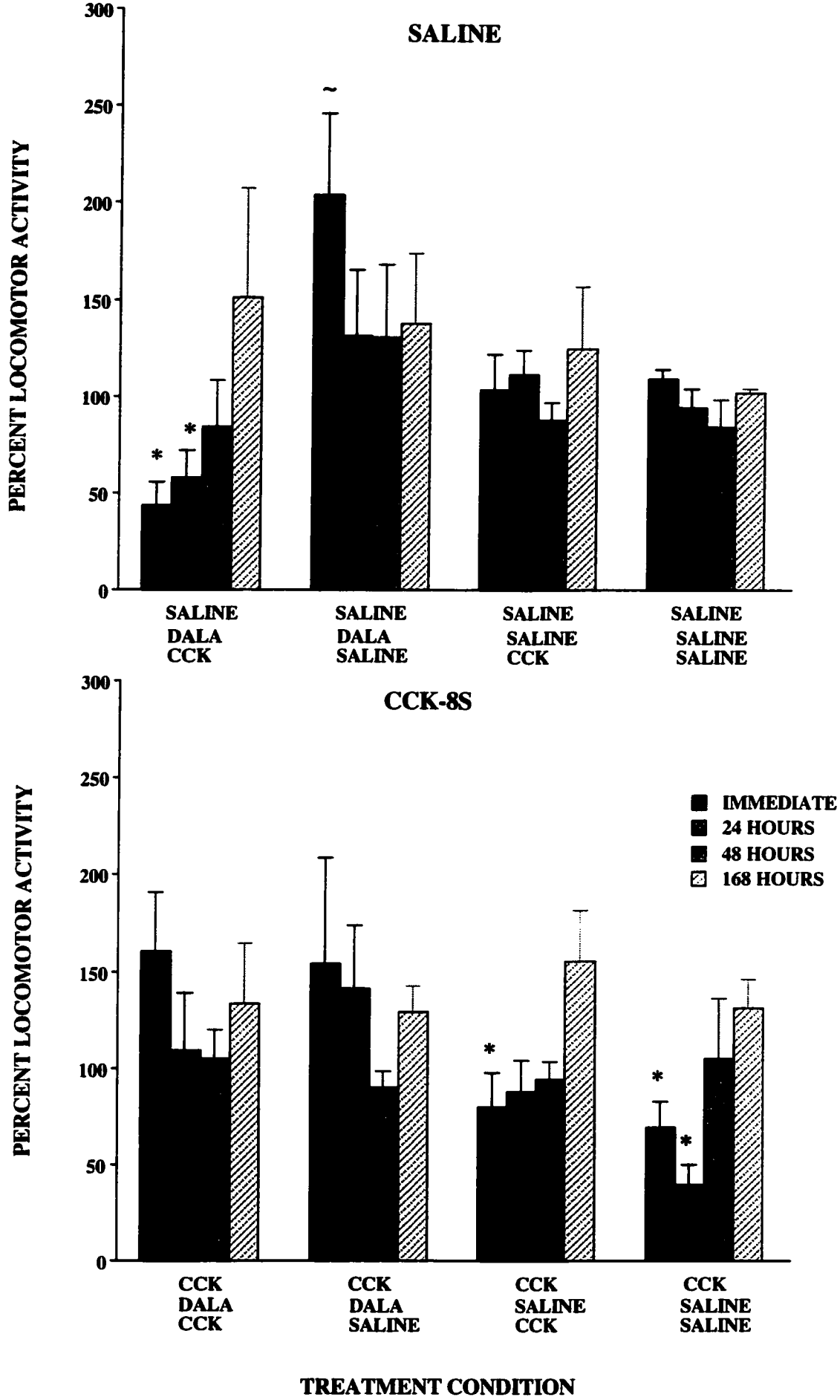
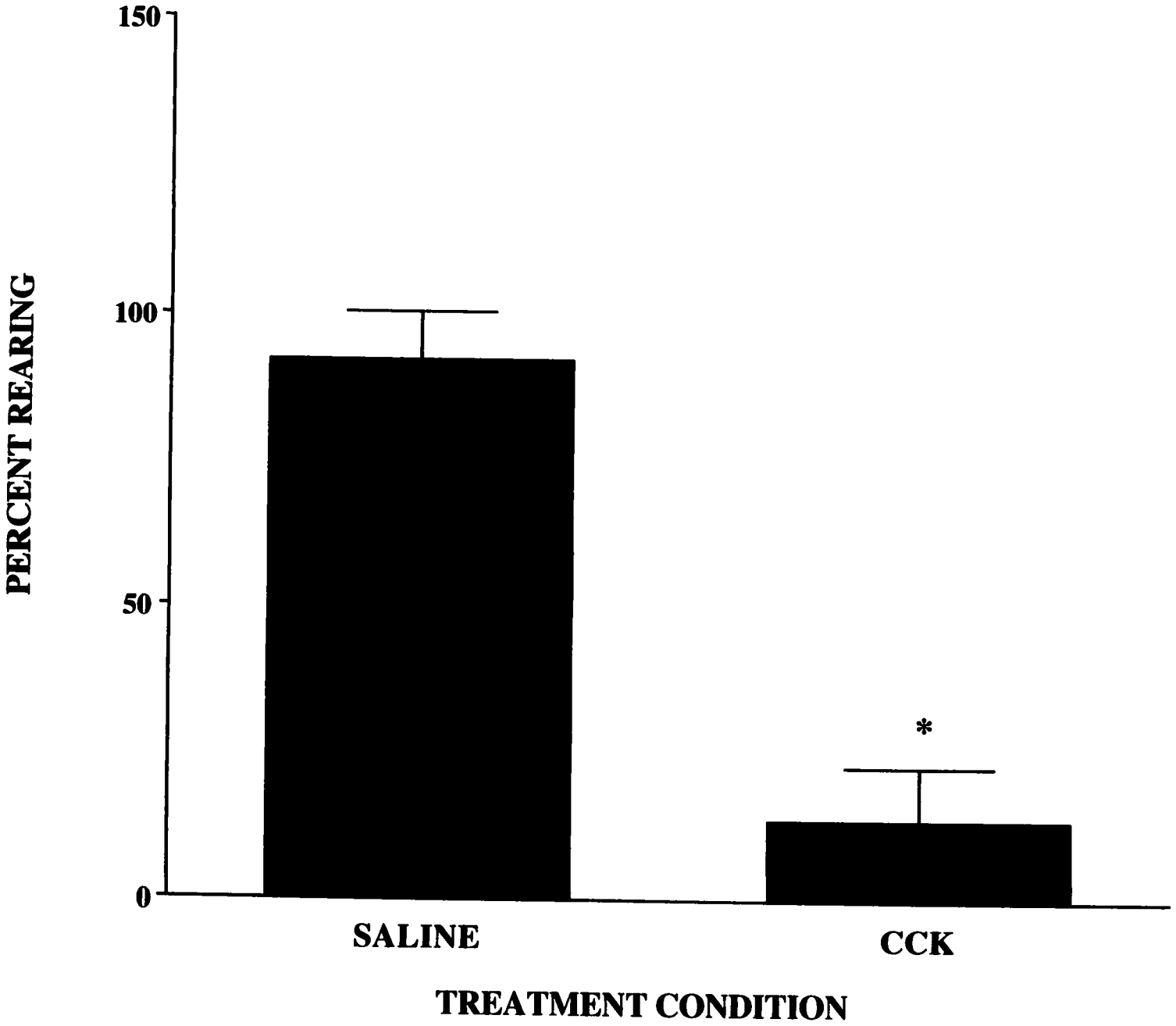

SALINE
PERCENT LOCOMOTOR ACTIVITY
300
250
200
150
100
50
0
~
*
*
SALINE
DALA
CCK
SALINE
DALA
SALINE
SALINE
SALINE
CCK
SALINE
SALINE
SALINE
CCK-8S
PERCENT LOCOMOTOR ACTIVITY
IMMEDIATE
24 HOURS
48 HOURS
168 HOURS
*
*
*
CCK
DALA
CCK
CCK
DALA
SALINE
CCK
SALINE
CCK
CCK
SALINE
SALINE
TREATMENT CONDITION

Figure 9.5: Rearing (± S.E.M.) expressed as a percentage of Baseline among mice immediately (5 minutes) following intraventricular saline (SAL) or 50 ng CCK-8S administration. Rearing was assessed for 45 minutes following either SAL or CCK challenge. The * depicts a statically significant ($p<.05$) decrease in rearing following 50 ng intraventricular CCK-8S relative to saline administration.


150
100
50
0
PERCENT REARING
*
SALINE
CCK
TREATMENT CONDITION

scores relative to SALINE/SALINE treated mice. Both SALINE/DALA and CCK/DALA showed elevated rearing scores relative to both CCK/SALINE and SALINE/SALINE treated animals. This effect was most pronounced during the immediate post-drug interval (see Figures 9.6 and 9.7).

Insert Figures 9.6 and 9.7 about here

CCK/SALINE treated mice exhibited enhanced rearing scores relative to CCK/DALA treated mice re-exposed to either SAL or 5 ng CCK, immediately, 24 and 48 hours post-drug 3 injection. SAL/SAL treated mice exhibited reduced rearing performance relative to SAL/DALA treated mice reexposed to SAL (see Figure 9.8).

Insert Figure 9.8 about here

DISCUSSION

The data of Experiments 8 and 9 revealed that administration of the mixed μ/δ enkephalin agonist DALA following CCK-8S administration blunted the protracted behavioural disturbances evoked by a challenge dose of CCK on ICSS and increased locomotor activity in paradigms assessing motivation and exploration. Mice challenged with CCK-8S demonstrated deficits in self-stimulation performance and elevated reward frequency thresholds from the dorsal aspects of the VTA up to 24 hours following 50 ng CCK-8S (i.e., CCK/SAL) administration while mice challenged with 50 ng CCK-8S did not exhibit

Figure 9.6: Rearing (± S.E.M.) expressed as a percentage of Baseline immediately (5 minutes) (A), 24 hours (B), 48 hours (C) or 168 hours (D) following SAL administration in mice previously treated with SAL or 50 ng CCK-8S. Rearing was assessed for 45 minutes following SAL challenge. The * depicts a statically significant ($p<.05$) decrease in rearing following 50 ng intraventricular CCK-8S/SAL relative to SAL/SAL administration at the identical test interval.

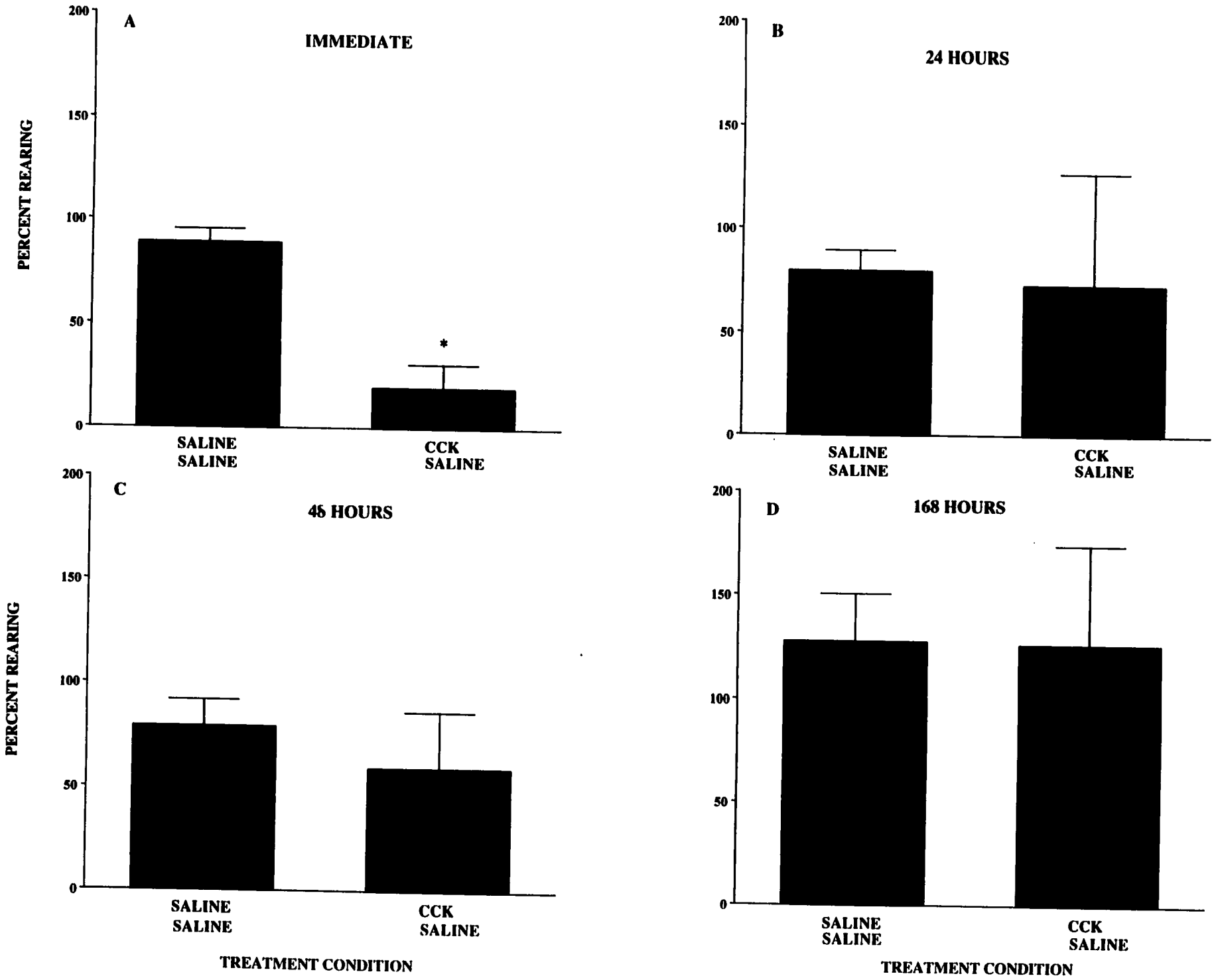

A
IMMEDIATE
B
24 HOURS
C
48 HOURS
D
168 HOURS
PERCENT REARING
PERCENT REARING
200
150
100
50
0
*
SALINE
SALINE
CCK
SALINE
TREATMENT CONDITION
TREATMENT CONDITION

Figure 9.7: Rearing (± S.E.M.) expressed as a percentage of Baseline immediately (5 minutes) (A), 24 hours (B), 48 hours (C) or 168 hours (D) following DALA administration among mice previously treated with SAL or 50 ng CCK-8S. Rearing was assessed for 45 minutes following DALA challenge. Note: ~ depicts a statically significant ($p<.05$) difference in rearing following SAL/DALA or CCK/DALA relative to SAL/SAL or CCK/SAL (Figure 9.6), respectively, at the identical test interval.

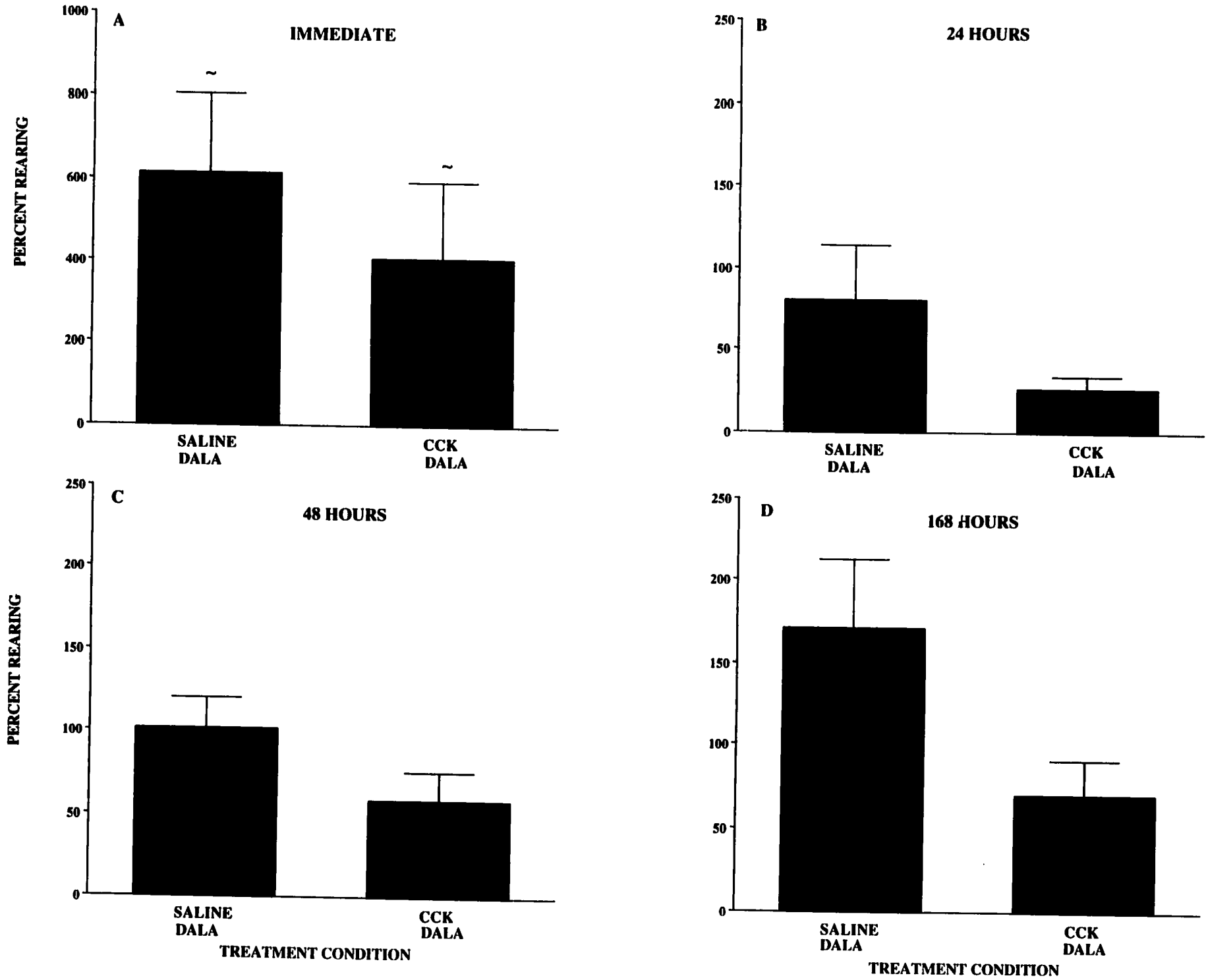
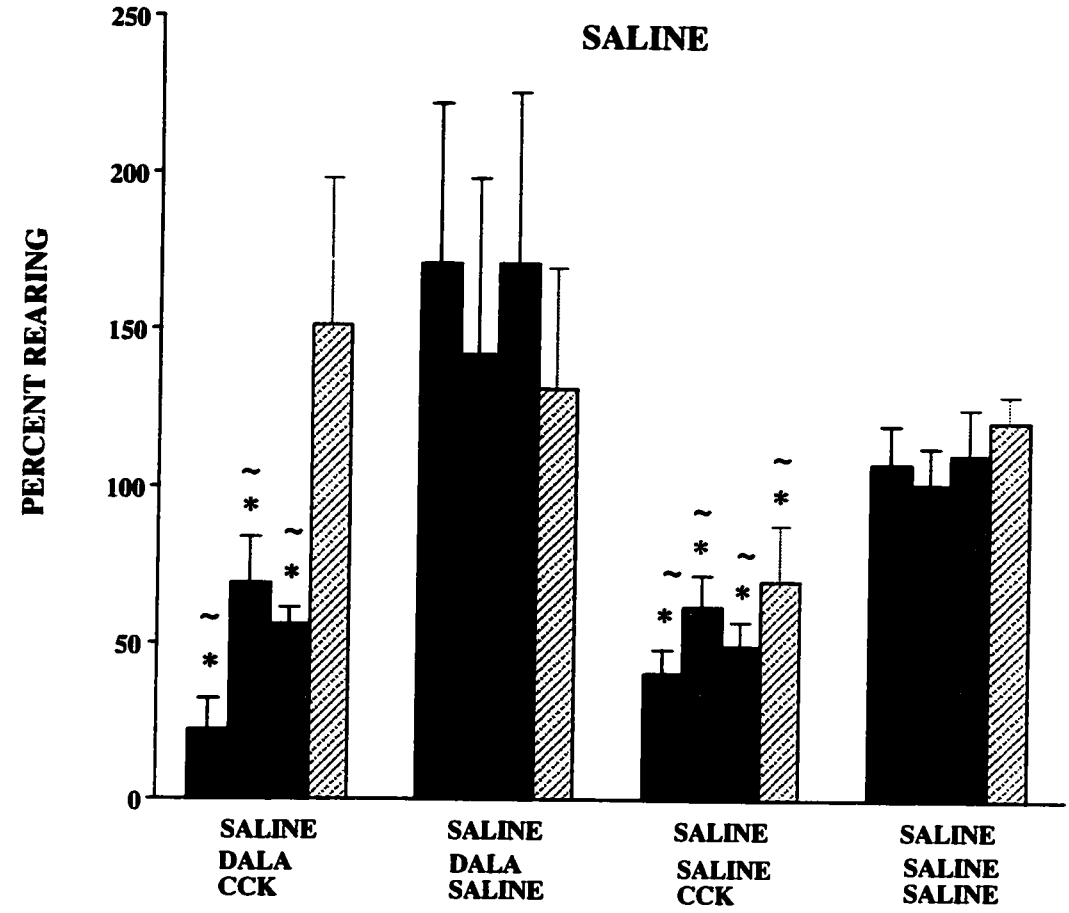
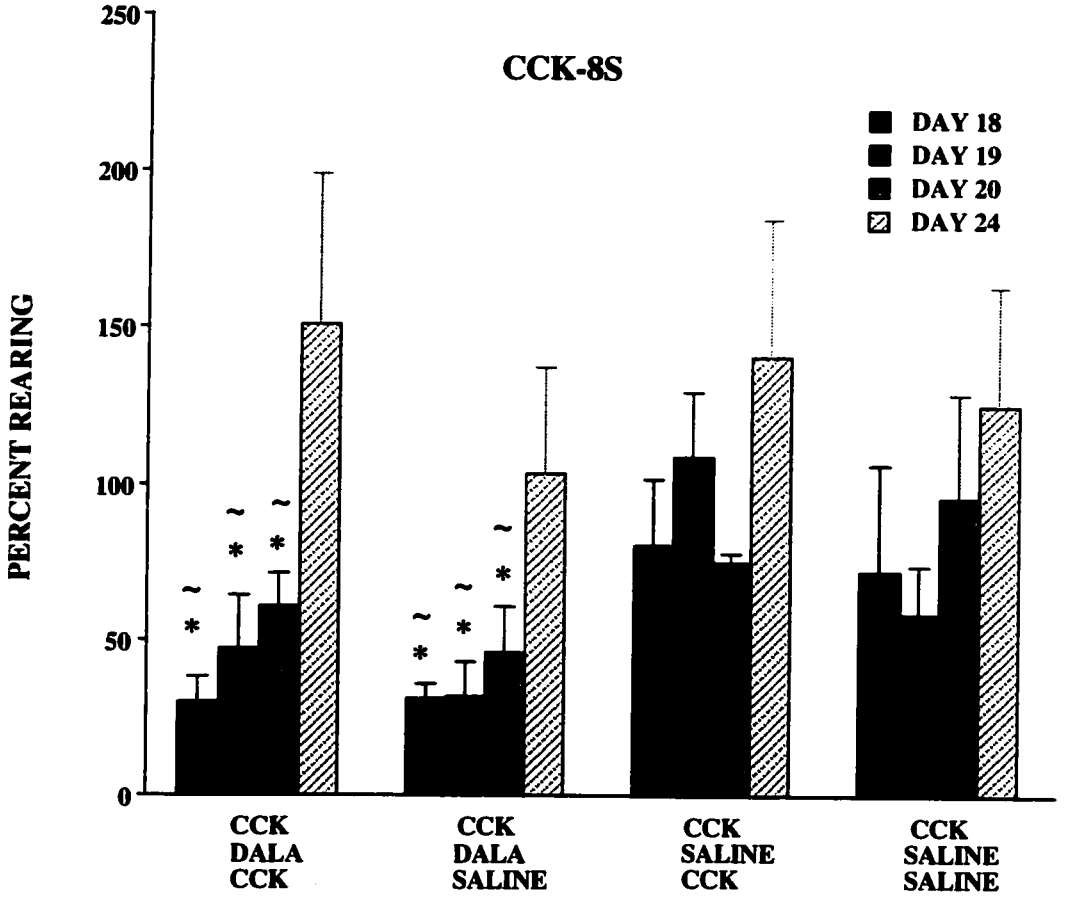

A
IMMEDIATE
PERCENT REARING
1000
800
600
400
200
0
SALINE DALA
CCK DALA
B
24 HOURS
250
200
150
100
50
0
SALINE DALA
CCK DALA
C
48 HOURS
PERCENT REARING
250
200
150
100
50
0
SALINE DALA
CCK DALA
TREATMENT CONDITION
D
168 HOURS
250
200
150
100
50
0
SALINE DALA
CCK DALA
TREATMENT CONDITION

Figure 9.8: Rearing (± S.E.M.) expressed as a percentage of Baseline immediately (5 minutes) (Day 18), 24 hours (Day 19), 48 hours (Day 20) or 168 hours (Day 24) following reexposure to SAL or 5 ng CCK-8S among mice previously treated with SAL (top panel) or 50 ng CCK-8S (bottom panel). Rearing was assessed for 45 minutes following SAL or CCK challenge. Note: * depicts a statically significant ($p<.05$) difference in rearing comparing treatment groups in the top panel with treatment groups immediately below it in the bottom panel (i.e. SAL/DAL/CCK with CCK/DALA/CCK) following reexposure to 5 ng intraventricular CCK-8S or saline. The ~ depicts a statically significant ($p<.05$) difference in rearing among treatment groups relative to SAL/SAL/SAL treated mice.


SALINE
PERCENT REARING
250
200
150
100
50
0
SALINE
DALA
CCK
SALINE
DALA
SALINE
SALINE
SALINE
CCK
SALINE
SALINE
SALINE


CCK-8S
DAY 18
DAY 19
DAY 20
DAY 24
PERCENT REARING
250
200
150
100
50
0
CCK
DALA
CCK
CCK
DALA
SALINE
CCK
SALINE
CCK
CCK
SALINE
SALINE
TREATMENT CONDITION

reductions in responding or elevations in frequency reward thresholds for brain stimulation from the ventral aspects of the VTA. The propensity of intraventricular CCK-8S administration to reduce ICSS from the dorsal but not the ventral aspects of the VTA was expected (see Hebb et al, 1998). The ventral aspects of the VTA support a dense opioid distribution compared to the dorsal aspects of the VTA (Pollard et al., 1989) which may contribute to the increased sensitivity of dorsal versus ventral VTA activity to stressor-induced anhedonia. In effect, intraventricular administration of CCK-8S produces anhedonic effects among animals responding for previously rewarding brain stimulation from the dorsal aspects of the VTA reminiscent of uncontrollable footshock. The purpose of the present experiment was twofold. First, to assess the anhedonic influence of the anxiogenic peptide, CCK on brain stimulation reward thresholds from the dorsal and ventral aspects of the VTA. Second, to evaluate the therapeutic and prophylactic propensity of enkephalin (i.e. DALA) to ameliorate the deficits in ICSS performance following 50 ng CCK-8S and blunt the reintroduction of anhedonia following subsequent CCK challenge at relatively protracted intervals. As the initial 50 ng CCK-8S dose did not influence responding for brain stimulation or reward thresholds from the ventral aspects of the VTA of mice, the assessment of μ/δ stimulation on CCK-induced behavioural deficits among mice immediately and following a subsequent CCK challenge was not evaluated.

The results of Experiment 8 revealed that intraventricular administration of 50 ng CCK-8S reduced self-stimulation performance and increased the stimulation frequency to effect half-maximal responding for brain stimulation from the dorsal aspects of the VTA. The reduction of ICSS performance was evident across all stimulation frequencies employed.

Deficits in responding for brain stimulation and increased frequency reward thresholds were noticeable immediately post-stressor (i.e., Day 1) and absent by 168 hours (i.e., Day 7) following CCK/SAL administration. It should be underscored that the CCK-8S dose employed did not provoke sedation as animals responding for brain stimulation from the ventral aspects of the VTA did not exhibit any deficits in ICSS responding. In fact, sedation has been reported to occur following CCK-8S doses exceeding 500 nanograms (Harro et al., 1990). The data of Experiment 8 is consistent with previous investigations which reported nanogram doses of intraventricular, intra-VTA and intra-accumbal CCK-8S reduced ICSS responding and increased reward thresholds from the VTA (Vaccarino & Koob, 1984; Singh et al., 1997) and the hypothalamus (Heidbreder et al., 1992) in rats. Increasing the current intensity reversed the effect of CCK-8S on VTA ICSS reward thresholds and restored baseline rates of responding in rats (Singh et al., 1997).

In the VTA, subpopulations of DA neurons containing CCK give rise to DA and DA/CCK projections. Microiontophoretic application of CCK in the VTA has been reported to decrease the firing rate of A10 DA neurons (Hommer et al., 1986) and administration of CCK-8S into the VTA increases A10 DA metabolite concentrations (Laitinen et al., 1990). There is a vast amount of data showing that CCK modulates DA regulated behaviour at the level of the nucleus accumbens (Crawley & Corwin 1994; Vaccarino, 1994). The main CCKergic projection to the nucleus accumbens arises in the ventral mesencephalon, specifically the VTA and pars compacta of the substantia nigra where CCK is colocalized with DA in 80-100% of SN neurons and 40-70% of VTA neurons (Lanca et al., 1998). In particular colocalized mesencephalic DA/CCK neurons project primarily to the caudal aspects of the nucleus accumbens, whereas the rostral accumbens receives independent CCK

and DA fibers (Hokfelt et al., 1980; Seroogy et al., 1989; Studler et al., 1981; Vaccarino & Rankin, 1989). It should be noted parenthetically that the data of the present investigation failed to reveal variations in self-stimulation performance or reward thresholds among CCK treated animals that could be attributable to an anterior-posterior gradient of electrode placements either in the dorsal or ventral aspects of the VTA. Intra-accumbal administration of CCK-8S caudally enhanced the rate of medial forebrain bundle (MFB) ICSS while a similar injection rostrally decreased MFB ICSS (DeWitte et al., 1987). The data of DeWitte et al. (1987) are consistent with the differential CCK_A and CCK_B receptor diversity in the rostral and caudal aspects of the nucleus accumbens. CCK_A activation promotes increased DA release from the accumbens and may thus underlie increased ICSS rates observed from the MFB. Dauge & Lena (1998) maintain that CCK_A receptors participate in the adaptive response to stress. Injections of proglumide, a mixed $CCK_{A/B}$ antagonist, in the caudal and rostral accumbens produced attenuation and potentiation of VTA ICSS, respectively (Vaccarino & Vaccarino, 1989). In the present investigation, it should be considered that CCK-8S influences both receptor subtypes. CCK_B receptors are predominant in the brain especially the cortex and limbic structures such as the hippocampus, the olfactory tubercle the amygdala and the nucleus accumbens (Hill & Woodruff, 1990). However, given the relative paucity of central CCK_A receptors compared to mesolimbic CCK_B receptor distribution and the observed decrease in ICSS behaviour it is reasonable to conclude that CCK_B receptor activation and subsequent decreased DA release in the nucleus accumbens may underlie the deficits of ICSS from the VTA following initial intraventricular 50 ng CCK-8S administration.

In addition to DA/CCK colocalization, opioid peptides in the VTA influence ICSS responding following stressor imposition. Interaction of endogenous opioid peptides with mesolimbic DA underlies motivated behaviour and ICSS in particular (Wise & Rompre, 1989). ICSS from the VTA in rats increases opioid release from the VTA, nucleus accumbens, amygdala and prefrontal cortex (Stein, 1993). Mu receptor density is conspicuous in the VTA, δ receptor sites predominate in the nucleus accumbens and in the prefrontal cortex μ and δ receptors are equally distributed. There are very few δ receptors in the VTA (Mansour et al. 1995 and 1997). However, δ receptor activation in the accumbens shell modulates accumbal DA and CCK (Svingos et al., 1999). The relative contributions of δ and μ receptor activation to motivated behaviour would be predicated on mesolimbic site-specific differences in receptor density as well as environmental stimuli which may modulate the release of variable neurotransmitter and neuropeptide systems with different affinities for the δ and μ receptor sub-types.

The deficits in self-stimulation performance and the increase in reward frequency threshold following 50 ng CCK-8S administration were not ameliorated in the immediate post-stressor interval with 1 μg DALA. In contrast to earlier investigations in this laboratory, central administration of DALA following induction of footshock associated ICSS alterations from the dorsal VTA provoked immediate recovery of responding for rate of ICSS. However, this therapeutic effect was transient and deteriorated 24 hours after initial exposure to uncontrollable footshock and was absent 1 week later (Maddeaux & Zacharko, 1992). In nonstressed rats, intra-VTA injection of the selective μ and δ agonists DAGO and DSTBULET increased self-stimulation rates from the lateral hypothalamus (Heidbreder et al.,

1992). However, in the present investigation, DALA administration following 50 ng CCK-8S administration did not restore ICSS response curves or reward thresholds in the immediate ICSS interval. In fact, DALA decreased ICSS responding at the higher frequencies (i.e., 60 Hz, 70 Hz and 80 Hz) and increased the threshold frequency for rewarding brain stimulation among mice previously administered SAL on Day 1. It will be recalled that ICSS increases opioid peptide release from the VTA and should be considered that the transient deficit in ICSS behaviour at higher stimulation frequencies as well as increased reward thresholds in SAL treated mice may be due to excessive behavioural activation.

Twenty-four hours post 50 ng CCK-8S injection, the ICSS pattern of DALA treated mice were indistinguishable from SAL/SAL treated mice. The delayed response of DALA to restore ICSS following CCK application may be mediated through changes in CCK receptor sensitivity or properties or CCK availability. Indeed, enkephalin is a competitive antagonist of CCK (Murphy et al., 1992) and likewise CCK a competitive antagonist of enkephalin (Gaudreau et al., 1990) interacting at the CCK_B receptor and δ-opioid receptor, respectively (Boteju et al, 1996; Rezvani et al., 1987). In addition, binding of CCK-8S with its receptor reduces the density and affinity of δ receptors in the cerebral cortex (Johnson et al., 1987) as well as μ receptors in whole rat brain as measured in vitro (Wang & Han, 1990; Zhang et al., 1993). It has been demonstrated in vitro that neuropeptidase degradation of CCK-8S and CCK-4 is in competition with the enzymatic degradation of leu-enkephalin (Deschodt-Lanckman & Bui, 1981; Rose et al., 1995, 1996). Among CCK/SAL treated mice, a decrease in ICSS responding from the VTA and elevated reward thresholds was in evidence 24 hours post-drug manipulations (i.e., Day 2) relative to the 3 other treatment groups indicative of a

somewhat protracted influence of CCK-8S on hedonic processes. In previous investigations in this laboratory, uncontrollable footshock and IL-2 administration decreased ICSS responding and elevated reward thresholds among mice responding for brain stimulation from the dorsal aspects of the VTA for up to 168 hours following stressor application (Zacharko et al., 1998; Hebb et al., 1998). The reestablishment of ICSS profiles in CCK treated mice 48 hours post-stressor relative to 168 hours following uncontrollable footshock may be reflective of stressor severity and associated neurochemical alterations. In effect, the neural processes underlying stressor and CCK alterations of self-stimulation performance may be distinguishable from one another.

Neurochemical and neuropeptide changes associated with CCK-8S administration may be subject to conditioning and sensitization such that reexposure to a very low dose of CCK-8S may reintroduce these amine and opioid changes accounting for protracted and exaggerated behavioural deficits. Indeed, mice previously administered 50 ng CCK-8S followed by SAL on Day 1 demonstrated exaggerated ICSS deficits and increased reward thresholds in response to 5 ng CCK-8S administration on Day 18. The ICSS deficits and subsequent increase in the frequency necessary to evoke 50% responding of maximal baseline values in CCK/SAL/CCK treated mice were apparent immediately (i.e., Day 18) , 24 (i.e., Day 19) and 48 (i.e., Day 20) hours post Drug 3 administration. ICSS response profiles among CCK/SAL/CCK treated mice were indistinguishable from SAL/SAL/SAL treated mice 168 hours (i.e., Day 24) following CCK-8S reexposure. The failure of SAL/SAL/CCK mice to demonstrate deficits in ICSS response profiles or increased frequency reward thresholds relative to SAL/SAL/SAL treated mice at any time point (i.e., Day 18, Day 19, Day 20 or Day 24) reestablished preliminary data that 5 ng of CCK-8S was a sterile dose of CCK-8S in

a reward paradigm. Among CCK/DALA/CCK treated mice, enhanced ICSS profiles and a subsequent decrease in the frequency necessary to evoke 50% responding of maximal baseline values relative to SAL/DALA/CCK treated mice was in evidence on Day 18 suggesting that previous activation of CCK receptors are necessary to evoke alterations in reward upon CCK reexposure.

Forty-eight hours following CCK-8S reexposure (i.e., Day 20), CCK/DALA/CCK mice exhibited enhanced ICSS response curves and decreased reward thresholds relative to the 7 other treatment groups. The delayed action of CCK-8S to evoke enhanced ICSS profiles in CCK/DALA treated mice may be indicative of an increased rewarding value of ICSS. It will be recalled that ICSS evokes opioid release from the VTA and DA release from the nucleus accumbens which sustains responding for brain stimulation. The contribution of mesolimbic CCK_A activation to enhanced ICSS profiles following a challenge CCK dose in mice previously administered CCK/DALA should also be considered. It will be recalled that CCK_A receptor activation in the nucleus accumbens is associated with increased accumbal DA release and increased ICSS performance from the MFB and VTA (DeWitte et al., 1987; Vaccarino & Vaccarino, 1989). To be sure, selective CCK_B agonists (e.g. CCK-4) must be employed to firmly establish if it is the CCK_A receptor that induces the increase in ICSS following CCK reexposure. These effects were short-lived as by 168 hours (i.e., Day 24) following CCK or SAL reexposure there were no apparent group differences in ICSS response curves or reward thresholds.

The observation that locomotor and rearing were reduced in CD-1 mice following intraventricular CCK-8S administration parallel previous investigations which report CCK-8S injected into the postero-median nucleus accumbens produced a marked decrease in

exploratory behaviour (Crawley and Corwin, 1994; Hirosue et al., 1992). In particular, CCK-8S activation of CCK_A receptors suppressed exploratory locomotion, rearing and grooming (Britton et al., 1989). CCK potentiation of DA-induced hyperlocomotion in the medial posterior accumbens is mediated by CCK_A receptors (Crawley, 1985) whereas CCK inhibition of DA-induced hyperlocomotion in the anterior accumbens appears to be mediated by the CCK_B receptor (Crawley, 1992; Dauge et al., 1989). In rats, administration of the CCK_A antagonist, devazepide blocked the augmented locomotor response to an acute dose of psychostimulant immediately (Vasar et al., 1991) and to a psychostimulant challenge dose 10 days following cessation of chronic d-amphetamine treatment (Desousa et al., 1999). In contrast, administration of the CCK_B antagonist L365-260 potentiated d-amphetamine-induced hyperactivity (Higgins et al., 1994). In the present experiment, CCK-induced locomotor deficits were apparent immediately (i.e., Day 1), 24 hours (i.e., Day 2) and 48 hours (i.e., Day 3) but not 168 hours (i.e., Day 7), following CCK/SAL administration. CCK-induced deficits in rearing among mice following initial Drug 1 administration were only apparent on Day 1. Not surprisingly, although in contrast to the ICSS behavioral profile, DALA increased locomotor and rearing activity in both SAL and CCK treated mice. The global hyperactivity induced by the intra-VTA injection of both δ and μ receptor agonists has been linked to activation of mesocorticolimbic DA pathways with subsequent increases in DA turnover in cortical and limbic sites (Kalivas & Bronson, 1985; Latimer et al., 1987). It should be noted parenthetically that activation of μ and δ receptors in the nucleus accumbens also contributes to enhanced locomotor activity (Schad et al., 1996; Svingos et al., 1999). Intra-VTA DALA administration dose-dependently increased DOPAC:DA ratio in DA

terminal regions, namely the nucleus accumbens, striatum and septum (Cador et al., 1988). In particular, stimulation of VTA μ-receptors produced marked increases in locomotor activity in rats (Kalivas & Abhold, 1987; Latimer et al., 1987 ; Vezina et al., 1987). Moreover, DALA injected into the VTA and nucleus accumbens increased locomotor activity in rats (Phillips et al., 1994). The μ-receptor agonist DAGO is a more effective stimulant of A10 DA neurons, and resultant hyperlocomotion, than the δ-receptor agonist DPDPE or the mixed μ/δ-receptor agonist DALA. In particular, the μ_1-receptor isoform modulates DA activity producing an increase in cortical and limbic DA metabolism and spontaneous motor activity (Cooper, 1991; Latimer et al., 1987).

Many studies report that CCK interacting with CCK_A receptors decreases locomotor activity (see Crawley & Corwin, 1994 for review) and that this effect suppresses the hyperlocomotion induced by endogenous enkephalins (Dauge et al., 1995). The rather nonspecific influence of DALA on locomotor activity and rearing in CCK/DALA and SAL/DALA treated mice imply that increased behavioural responsivity following DALA is not CCK dependent. Indeed, CCK_B activation reduces DA activity in the nucleus accumbens (Crawley & Corwin, 1994; Heidbreder et al, 1992; Marshall et al., 1991) and DALA increases DA activity in mesolimbic sites (Churchill & Kalivas, 1992; Kalivas & Duffy, 1990). For example, SAL/DALA treated mice exhibit increased locomotor and rearing behaviour relative to CCK/DALA treated mice suggesting a compensatory mechanism of activation on accumbal DA metabolism. Peripheral administration of BC264, a specific CCK_B agonist induced hyperexploration in a novel environment and increased levels of met-enkephalin immunoreactivity (Met-LI) in the anterior part of the nucleus accumbens. The

effects of BC264 on locomotor activity and on the extracellular levels of Met-LI in the anterior part of the nucleus accumbens were antagonized by local injection of the δ antagonist naltrindole in the anterior part of the nucleus accumbens as well as systemic administration of the CCK_B antagonist L-365,260 (Dauge et al., 1999).

Locomotor acrivity in mice previously exposed to CCK-8S (50 ng) and reexposed to a milder form of the initial stressor CCK-8S (5 ng) was influenced by an intervening dose of 1.0 μg DALA. Reexposure to either SAL or 5 ng CCK-8S increased locomotor activity in CCK/DALA treated mice relative to CCK/SAL treated mice in the immediate test interval. These data suggest that contextual cues associated with previous DALA administration may contribute to the enhanced locomotor activity. Indeed, behavioural exaggeration was evident immediately following CCK challenge and dissipated by 24 hours. It appears that mildly aversive stimuli may increase the association of salient cues and environmental context. Interestingly in SAL/DALA treated mice reexposure to 5 ng CCK-8S induced deficits in locomotor activity immediately and 24 hours following drug administration. It appears that these animals are more sensitive to the anxiogenic properties of CCK-8S. In contrast to locomotor activity, reexposure to CCK or SAL decreased rearing in CCK/DALA treated mice. At first glance, the reduction of rearing behaviour following reexposure to CCK or SAL in CCK/DALA treated mice appears conspicuous. However, it should be emphasized that locomotor activity and rearing were evaluated concurrently and an exaggerated increase in locomotor activity would not necessarily favor a simultaneous increase in rearing. Moreover, SAL/SAL/CCK and SAL/DAL/CCK rearing behaviour is decreased relative to SAL/SAL/SAL mice suggesting that rearing behaviour is more sensitive to CCK and DALA does not effect

this behavioural change. In effect, the neural mechanisms that underlie activity are not congruent with those that subserve rearing behaviour.

The neurocircuitry underlying contextual conditioning includes the nucleus accumbens (Riedel et al., 1997) and the VTA (Nader & LeDoux, 1999). Ordinarily, μ-receptor activation on GABA interneurons prompted by stressor presentation disinhibits mesolimbic DA and favors sensitization (Kalivas & Abhold, 1987). Increased δ_1-receptor activation increased locomotor activity among CD-1 mice encountering environmental stimuli which resembled the contextual cues of previous experiences. These data suggest that prior δ_1-receptor activation over compensates for behavioural depression induced by future stressor encounters (Hebb et al., 1997). Endogenous δ_1-receptor activation has also been reported to modulate attentional variables attending environmental stimuli (Hernandez & Watson, 1997). It will be recalled that injection of non-anxiogenic (e.g., assessed by the elevated plus maze) doses of CCK-8S (1 ng) into the central amygdaloid nucleus enhanced performance of mice trained to avoid footshock (Huston et al., 1998). The facilitatory effect of systemic CCK-8 on retention performance was blocked by joint intra-amygdaloid administration of opioids suggesting that the effects of CCK on anxiety as well as mnemonic processes are mediated through the amygdala (Huston et al., 1998). Clearly, the distribution of μ and δ receptors in mesolimbic sites contributes to the emerging profiles of behavioural sensitization. Moreover, such neural processes may underlie coping (Goodwin & Barr, 1997). Conceptually, it is conceivable that CCK-8S preserves the memory of a CCK encoded event or stressor and central μ_1 and δ_1 receptor activation interferes with the encoding of such events. Taken together, alterations in mesolimbic CCK availability define mild anxiety states. In particular, a

μ_1 and δ_1/ CCK_B neural interface may diminish anxiety and motivational loss accompanying stressor exposure, detract from the saliency of the stressful event and promote coping. Such processes may alter long-term responsivity of organisms to future stressor encounters.

In summary, the present experiments provide behavioural evidence for a CCK-opioid interface in the modulation of anxiety and motivation. In particular, the sensitizing effect of a previous anxiogenic dose of CCK-8S to a CCK challenge dose is averted by μ/δ receptor activation in ICSS. These data parallel other studies evaluating the propensity of traditional anxiolytic and antidepressant agents to counteract the sensitization process. In attempting to account for the differing ability of intraventricular administration of DALA following CCK-8S administration to increase ICSS behaviour and not locomotor/rearing following a previously established sterile dose of CCK-8S, it should be considered that central administration of CCK-8S and/or DALA incited behavioural change by acting at multiple central sites. Moreover, while the CCK_B/δ-receptor activation and related DA alterations may underlie deficits in ICSS, CCK_A/μ-receptor activation may induce alterations in exploration. Nevertheless, central opioid peptides in conjunction with CCK accommodate the demand characteristics of stressor experience, entrain associations between stressors, modulate vigilance and attentional sets and allow manifestation of immediate or delayed behavioural effects. There is reason to suspect that the interplay between CCK and opioid peptides contributes to motivational alterations in human subjects (Perez-Costillas et al., 1997; Shlik et al., 1997). In conclusion, CCK and enkephalin appear to have an opposite role in the behavioural response of mice in motivational, locomotion and exploration paradigms. Immediate activation of central μ and δ receptors appears to have delayed therapeutic

influence over CCK-reduced behavioural change and prophalactically compensates for the subsequent influence of future stressor encounters. The propensity of enkephalin to increase mood and affect and detract from the saliency of the stressor experience suggests increased enkephalin availability within the mesencephalon ameliorates protracted CCK-induced motivational alterations. It would be appropriate to target pharmacological manipulations which effect alterations in mesolimbic opioid activity in the treatment of panic and anxiety forms in which alterations in central CCK are a prominent characteristic.

GENERAL DISCUSSION

The present series of experiments evaluated the conjoint pattern of mesolimbic CCK and enkephalin alterations in discrete central sites induced by psychogenic stressor exposure and the influence of such manipulations on fear, anxiety, startle and reward at several post-manipulation intervals. In particular, an acute exposure to a mild psychogenic stressor (e.g., novel odors, predator odors) enhanced CCK availability within the mesencephalon, mesocortex and subregions of the amygdala conducive to conditioning and sensitization. The evaluation of fear and anxiety in CD-1 mice with psychogenic stressor manipulations were paralleled by pharmacological assessment of the anxiogenic influence of central CCK within these identical paradigms. To date, behavioural sensitization accompanying CCK challenge in paradigms that assess motivation and anxiety have not been attempted. Such investigations necessitate assessment of behavioural responsivity of animals to previously non-anxiogenic doses of CCK and a temporally relevant inter-CCK interval.

In brief, exposure of CD-1 mice to a) a neutral odor in a novel environment (CO), b) a pungent odor (butyric acid) in the home cage, c) a novel environment lined with soiled rat shavings (PO) or d) the predator odor, TMT presented in the home cage induced anxiety in

the light dark box immediately following stressor presentation. Mice exposed to a) the neutral odor (CO) and rat odor (PO) in a novel environment or b) butyric acid and TMT displayed decreased latency to enter the dark chamber of the light dark box and spent less time in the light portion of the apparatus relative to home-caged or saline treated mice, respectively. Behavioural deficits in the light dark box were accompanied by elevated CCK mRNA levels from the medial prefrontal cortex (mPFC), ventral tegmental area (VTA) and the basolateral (BLA) nucleus of the amygdala in mice exposed to the novel environment or rat odor relative to home-caged mice. In particular, enhanced BLA CCK mRNA levels following predator exposure were transient appearing immediately following stressor imposition and returning to control values 30-60 minutes following stressor termination. In contrast, CCK mRNA levels in the VTA and mPFC appeared within 30 minutes of stressor exposure and persisted for 4 hours following stressor termination. In comparison, home-cage exposure of mice to butyric acid or TMT odor was associated with increased CCK mRNA in the VTA and mPFC or only the mPFC, respectively. Anxiety in the light-dark paradigm, however, was paralleled by increased CCK mRNA expression in the basolateral nucleus of the amygdala in mice exposed to butyric acid or TMT. At first glance it might appear that rat odor and TMT produce different forms of anxiety effected by alterations in CCK mRNA from the mPFC and VTA. It should be emphasized, however, that the association of anxiety in the light dark box and enhanced VTA and mPFC CCK mRNA among animals exposed to rat odor or a novel environment may be due to lack of inclusion of an appropriate control group for light dark box exposure. In contrast, the medial amygdaloid nucleus hosted similar increases in CCK mRNA to both novelty (i.e., novel environment and butyric acid) and both predator odors (i.e., rat and fox) relative to home-caged or saline treated mice, consistent with a role of

this nucleus in behavioural arousal and social memory (Kollack-Walker & Newman, 1995; Vochteloo & Koolas, 1987). These data suggest that CCK activity in the BLA may be involved in the initiation of anxiety-like behaviour while mesocortical CCK may sustain stressor-associated cues over longer periods of time following predator odor, in particular.

In addition to anxiety in the light dark box, mice exposed to TMT displayed enhanced levels of freezing relative to saline or butyric acid treated mice. Among mice exposed to TMT, increased levels of freezing were associated with subsequent anxiety in the light-dark box as indicated by reduced time spent in the light portion of the light dark box. Enhanced freezing or fear levels among TMT exposed mice were associated with enhanced neural activation in ENK neurons in the nucleus accumbal shell (i.e. FRA/ENK) and decreased enkephalin mRNA from the central amygdaloid nucleus. Moreover, anxiety in the light-dark box was associated with increased FRA and decreased ENK mRNA in the accumbal shell and core, respectively. Anxious mice displayed increased enkephalin mRNA in the basolateral (BLA), central (CEA) and medial (MEA) amygdaloid nuclei relative to their non-anxious counterparts. In the BLA and CEA, anxious mice also displayed increased FRA/enkephalin following odor exposure. In contrast to the anxiogenic influence of butyric acid and TMT on behaviour in the light dark box, a 10 minute TMT or butyric acid exposure was ineffective in altering reward thresholds among mice responding for brain stimulation from the VTA. Taken together, acute psychogenic stressor exposure was associated with discrete episodes of fear and anxiety in the startle and light-dark paradigms occasioned by alterations in CCK and enkephalin from distinct mesolimbic sites immediately following stressor imposition.

It should be emphasized that anxiety in the light dark box following psychogenic stressor exposure was conducted in independent groups of mice. Mice assessed in the light-

dark box 24, 48 or 168 hours following psychogenic stressor exposure did not display anxiety-like behaviour. At this juncture, it is not clear whether repeat psychogenic exposure would produce an exaggeration of anxiety in mice relative to control mice. Indeed, subsequent exposure of rats and mice to these identical paradigms in the absence of stressor applications effects an anxiety "form" (i.e., phobia) which is resistant to the anxiolytic influence of either diazepam or midazolam (File & Zangrossi, 1993). In contrast to light dark box behaviour, exposure of CD-1 mice to 2, 5 or 10 minutes of rat odor or 2 or 10 minutes of TMT increased acoustic startle in mature mice relative to mice in control conditions for up to one week following predator exposure. Interestingly, a lower ^{3}H-CCK-8 hippocampal binding density (Harro & Oreland, 1992), as well as decreased CCK mRNA in the hypothalamus and cerebral cortex (Miyasaka et al., 1995) and increased CCK concentrations in the cerebral cortex (Ohta et al., 1995) have been detected among rats 18-29 months of age relative to younger animals (i.e., 2-10 months). In contrast to the behavioural repercussions of clean shavings and butyric acid on anxiety in the light-dark box, mice exposed to the novel odor did not exhibit enhanced startle relative to home-caged mice. Among mice exposed to 10 minutes of butyric acid enhanced startle was evident 168 hours post-odor exposure reminiscent of the 2 minute rat odor exposure. Interestingly, the 2 minute exposure of mice to rat odor produced a different profile of startle reactivity (i.e., anxiety profile) compared to a 2 minute TMT exposure. Mice exposed to 2 minutes of TMT in the home cage displayed elevated startle relative to mice exposed to butyric acid or saline, immediately, 24 hours and 48 hours following odor exposure. In effect, experience of predator odor in the home cage may represent a more severe stressor or more relevant threat to the organism. Nevertheless, CCK mRNA elevations in the basolateral nucleus, mPFC and VTA following predator odor may increase vulnerability to

ensuing stressor encounters. However, acoustic startle rather than the light-dark paradigm may be more applicable for determining lasting increases in conditioned anxiety with repeated stressful (i.e., startle stimulus) encounters. Indeed the central amygdaloid nucleus is conspicuously more sensitive to CCK-4 relative to the prefrontal cortex or the nucleus accumbens in the startle paradigm (Vaccarino et al., 1997). However, a 2-minute exposure of mice to fox odor increased startle immediately while exposure of mice to 2 minutes of rat odor increased startle 1 week following odor presentation. Evidently, contextual cues associated with odor exposure influence the expression and emergence of behavioural reactivity in the acoustic startle paradigm.

In the startle paradigm, central administration of 50 ng CCK-8S and 5 μg Boc-CCK4 increased acoustic startle dependent upon the previous stressor history of the animal. Systemic administration of 5 μg of Boc CCK-4 potentiated startle amplitude in previously shocked mice to the conditioned stimulus (Light + Tone). The potentiated startle effect was maintained for 168 hours post-drug administration. Indeed, the difference in the startle scores of mice in the Light + Tone treatment condition relative to mice in the Tone alone group was most robust 168 hours following systemic Boc CCK-4 administration. In contrast, intraventricular administration of 50 ng CCK-8S enhanced startle amplitude 48 hours and 168 hours post-drug manipulation in CD-1 mice independently of prior stressor history. In the light-dark task and elevated plus maze, low doses of the CCK-8S agonist, ceruletide or the CCK_B agonist, pentagastrin are only anxiogenic among mice previously exposed to a stressful, overcrowded housing conditions. Substantially elevated doses of ceruletide and pentagastrin are required to induce comparable levels of anxiety in rats and mice housed in non-crowded

conditions (Harro et al., 1993). Furthermore, investigations in non-human primates indicate that intravenously administered CCK-4 dose dependently increased fear and defensive behaviours according to the baseline anxiety scores of animals and their social hierarchical position (Palmour et al., 1992). Apparently, antecedent environmental experiences interact with the nature of subsequent pharmacological challenges in provoking anxiety.

A role for the amygdala in eliciting fear potentiated startle has been empirically demonstrated in infrahuman (Davis, 1992) and human (Angrilli et al., 1996) subjects. For example, bilateral ablation of the central nucleus of the amygdala prevented sensitization of the startle reflex following repeated administration of footshock (Hitchcock et al., 1989). Anatomical investigations reveal that amygdaloid innervation derived from the VTA and prefrontal cortex may be involved in fear associated conditioning and anxiety. For example, efferent projections from the central and basolateral nuclei of the amygdala to the VTA may mediate conditioned fear associated increases in prefrontal DA turnover and increase vigilance, operationalized by increased cortical EEG activity (Davis, 1992; Davis et al., 1991). Moreover, the central and basolateral amygdaloid nuclei (Gelsema et al., 1987; Soltis et al., 1997) and the VTA (Chen et al., 1997; van den Buuse, 1998) provide prominent parabrachial innervation and accordingly may influence cardiovascular responsivity to environmental challenge. More specifically, alterations in amygdaloid CCK and DA activity may promote enhanced behavioural responsivity to stressor-associated cues. For example, administration of the CCK_B agonist pentagastrin into the central amygdaloid nucleus increases (Frankland et al., 1997) while systemic administration of the CCK_B antagonist, L-365, 260 dose dependently reduced (Josselyn et al., 1995) fear potentiated startle in the rat. Likewise, intra-VTA infusion of quinpirole, a $D_{2/3}$ agonist, attenuated fear potentiated startle in rats which has

suggested a role for DA in the promotion of fear-motivated behaviour (Borowski & Kokkinidis, 1996). At this juncture, it is not clear whether attenuation of the enhanced startle response followed from an attenuation of central DA activity via stimulation of DA autoreceptors in VTA and/or concomitant alterations of central CCK activity. Nevertheless, provisional consideration of the argument that exacerbation of fear-potentiated startle follows from alterations of central mesolimbic DA/CCK activity from a sensitization and conditioning perspective is an appealing one.

It has previously been demonstrated in this laboratory that intraventricular administration of CCK-8S dose dependently (0, 5 ng, 25 ng and 50 ng) increased anxiety in the light-dark box immediately following drug administration in non-shocked CD-1 mice (MacNeil et al., 1997). The notion that CCK incites ICSS deficits from the VTA (Vaccarino & Koob, 1984) or the μ/δ receptor complex may promote behavioural change is not novel (Zacharko et al., 1998) although consideration of μ/δ receptor activation in the alleviation of CCK-associated reductions of self-stimulation performance from the mesencephalon have not previously been considered. Specifically, we established that (a) intraventricular administration of 50 ng CCK-8S induced protracted deficits in ICSS from the dorsal VTA as well as locomotor and rearing perturbations and (b) administration of the enkephalinase resistant enkephalin analog DALA blunted the motivational and exploration deficits associated with a challenge dose of CCK given 18 days following initial challenge. The therapeutic influence of μ/δ receptor activation on locomotor and rearing was paralleled, albeit delayed, by CCK-induced disturbances in self-stimulation from the dorsal VTA. Moreover, a CCK challenge dose was effective in exacerbating deficits in self-stimulation reward and

exploration in mice previously challenged with CCK. The prophylactic influence of DALA was immediate in both paradigms supporting the propensity of μ/δ receptor activation in behavioural sensitization.

The mesolimbic circuitry underlying contextual conditioning and sensitization has been linked to the nucleus accumbens (Riedel et al., 1997) and the VTA (Nader & Ledoux, 1999) and the mesencephalic NMDA-GABA-DA-CCK interface. Mesencephalic and mesolimbic opioid receptor density appears to coincide with somatodendritic DA, CCK and GABA interneuron density (Kalivas, 1993; Mansour et al., 1988). Notably, central and basolateral nuclei amygdaloid neurochemical perturbations associated with benzodiazepine-GABA receptor variations including alterations in GABA and glutamate (Davis et al., 1994; Kunas & Varga, 1995; Soltis et al., 1997; Walker & Davis, 1997) most likely potentiate the efficacy of anxiolytic agents. It should be underscored that μ receptors have been identified on both GABA and DA- containing neurons and δ receptors have been detected on afferent inputs to GABA neurons in the VTA (Kalyuzhny & Wessendorf, 1997). Such anatomical data provide a putative framework for a μ/δ interaction in the VTA that may impact on stressor-associated behavioural deficits. It has reported that the release of enkephalin within mesolimbic sites diminishes the impact of the stress response by attenuating an array of physiological responses including emotional and affective states (Dziedzicka-Wasylewska & Papp, 1996). It should be considered that acute and chronic administration of the antidepressant/anxiolytic agent imipramine, effects neurochemically distinct profiles of met and leu enkephalin release within the VTA and nucleus accumbens (Dziedzicka-Wasylewska & Rogoz, 1995). Moreover, chronic imipramine treatment promoted the expression of the μ-

receptor in the hippocampus and frontal cortex of the rat (de Gandarias et al., 1998) and inhibited the enkephalin-degrading aminopeptidase in a concentration-dependent manner in rat brain (De Gandarias et al., 1997; Gallego et al., 1998). The variable sensitivity of central mesocorticolimbic sites to antidepressant regimens is consistent with data outlining differential responsivity of mesocorticolimbic sites to stressor imposition (Zacharko & Anisman, 1991). These data may extend to CCK induced anxiety and motivational deficits in both infrahuman and clinical subjects (Janssen & Arntz, 1996; Smadja et al., 1997). Taken together, increased enkephalin availability may represent a modulatory system in stress adaptation in both infrahuman and human subjects and avert exaggerated behavioural and neurochemical cascades associated with reexposure to stressors or cues previously associated with initial stressor presentations.

It should be considered that intraventricular administration of a bolus 50 ng CCK-8S dose, and not predator odor exposure, per se, incites anxiety states which are effective in attenuating the rewarding properties of ICSS. These findings are intriguing given the wealth of data implicating stressor severity, neurochemical change and the induction of pathology (Bifulco et al., 1998; Brown, 1993). In effect, pharmacological stressors may dose dependently enhance the manifestation of behavioural features characteristic of severe anxiety conditions while application of milder stressors would induce more subtle variations in affective and motivational state occasioned by mesolimbic CCK and ENK release profiles. To be sure, future experiments should assess anxiety and concomitant CCK/ENK variations within specific mesolimbic sites in response to reexposure to predator cues at protracted intervals following initial predator odor encounters. In effect, anhedonia and anxiety may undergo concurrent exacerbation following stressful experiences. However, mild stressor

experiences incite initial indices of anxiety and appear to only have weak influence on anhedonia (c.f., ICSS and sucrose consumption, Nielsen et al., 2000). While it has been well documented that recurrent anxiety episodes provoke depressive episodes, repeated anxiety episodes together with the affective disturbance of depression may evoke severe anxiety conditions including panic (see Keller & Hanks, 1993 for review). Certainly, subsequent experiments should include repeated exposure of mice to predator odors to determine if such intermittent, chronic anxiety episodes are effective in attenuating rewarding brain stimulation. These findings have profound implications for the etiology of anxiety disorders and the treatment thereof.

Severe anxiety syndromes, including panic and post-traumatic stress disorder are associated with increased sensitivity to the panicogenic properties of yohimbine (Albus et al., 1992; Southwick et al., 1993, 1997, 1999) and CCK-4 (Bradwejn et al., 1990; Kellner et al., 1998). Moreover, anxiety experience per se appears to be preserved. Indeed, administration of yohimbine or CCK-4 among panic patients elicits subjectively perceived anxiety-like effects reminiscent of those ordinarily associated with panic (Albus et al., 1992; Bradwejn et al., 1990), while promoting combat related 'flashback' memories which defined the anxiety and panic of individuals with post-traumatic stress disorder (Eriksson et al., 1989; Southwick et al., 1999). The propensity of CCK in eliciting respiratory and cardiovascular perturbations attending anxiety and panic experience is well documented (see Zacharko et al., 1995 for review). Systemic CCK induced panic is associated with increased adrenocorticotropin hormone (ACTH) and cortisol release as well as increased heart rate and blood pressure (Abelson & Liberzon, 1999; Reisine & Jensen, 1986) which have been attributed to concomitant alterations of central NE activity (Jerabek et al., 1999). Furthermore, D-Ala^2, N-

Me-Phe^4, Gly-Ol^5-enkephalin (DAGO) induced μ-receptor activation in the parabrachial nucleus attenuated the impact of relatively severe neurogenic stressors as revealed by cardiovascular attenuation (Kiritsy-Roy et al., 1986; Marson et al., 1989; Sun et al., 1996; Wisniewska & Wisniewski, 1996). In effect, brain specific alterations in CCK and enkephalin availability may contribute to some unique aspects of anxiety (Bystritsky & Shapiro, 1992; Ley, 1996).

Individuals with panic disorder or post-traumatic stress disorder also display augmented levels of aggression (Korn et al., 1997; Southwick et al., 1999). For example, some panic patients report an increased incidence of panic-associated suicidal ideation, engage in property destruction, initiate physical assaults and may exhibit homicidal tendencies (Korn et al., 1997). Moreover, the incidence of aggressive episodes among patients with post-traumatic stress disorder appeared to increase with attending symptom severity (McFall et al., 1999). Interestingly, mice lacking delta opioid receptors (Filliol et al., 2000) or prepro-enkephalin-derived peptides display increased fear and anxiety in novel environments and increased aggression towards conspecifics (Diaz & Asai, 1990; Kieffer, 1999; Konig et al., 1996). In a similar vein, naltrexone has been reported to increase aggression and blood pressure in an individual with post-traumatic stress disorder (Ibarra et al., 1994) and induce panic attacks in individuals with panic disorder (Maremmani et al., 1998). Aggression in infrahuman subjects, individuals with panic disorder and post-traumatic stress disorder has been associated with increased sensitivity of 5-HT$_{1A}$ receptors in the raphe nuclei and hypothalamus (Korn et al., 1997; Southwick et al., 1999). Met- and leu-enkephalin, β-endorphin and CCK are colocalized with 5-HT in the caudal raphe nuclei (Kachidian et al., 1991; Wang et al., 1998).

These data suggest that increased anxiety-like behaviour may be associated with a disinhibition of 5-HT in raphe nuclei (Albert & Walsh, 1982; Ferris et al., 1997; 1999). It should be underscored that the severity of the stressful experience as defined by organismic, experiential and stressor characteristics may elicit protracted alterations of DA, NE and 5-HT availability which define the expression of specific anxiety indices. In particular, severe stressor experiences in enkephalin deficient mice may be punctuated by aggressive display prompted by CCK/5-HT interaction and panic-like behaviour effected by CCK/NE interfacing. Consistent with such an analysis, mild stressor imposition would elicit mild anxiety in animals in infrahuman anxiety paradigms occasioned by perturbations of CCK expression in mesolimbic DA sites. Such data are in evidence in the light-dark paradigm following mere exposure of animals to a novel odor or novel environment.

Clearly, prominent behavioural correlates of central CCK and ENK activation are in evidence following stressor manipulations. In particular, CCK and ENK availability in diverse brain areas associated with anxiety-like behaviours (e.g., CCK and ENK within the amygdala may contribute to affective aspects of an anxiety provoking situation) points to the antagonistic role of these peptides in many of the responses of an organism following stressor impositions. It will be recalled that CCK availability is linked to colocalization of other neurotransmitters in distinct central sites which suggests that CCK may modulate (a) different aspects of anxiety, including anticipatory reactions to anxiogenic stimuli, (b) variations in cognitive arousal and vigilance and (c) sensitization and conditioning of behaviour and central neurochemical activity (e.g., DA, ENK, glutamate). This apparent overlap of functions may provide an organism not only with a redundancy of response, but more importantly the ability to manufacture diverse reactions in response to anxiogenic situations,

including coping. Likewise, multiple anxiogenic agents and putative neurotransmitters or neuromodulators in the mesencephalon, the limbic system as well as prefrontal cortex and brain stem sites would appear to participate in the promotion or alleviation of anxiety-like symptoms and mood alterations. Clearly, the distribution and interaction of enkephalin with CCK in mesocorticolimbic sites contributes to the emerging profiles of behavioural sensitization. To be sure, forthcoming investigations should assess the influence of such psychogenic and pharmacological manipulations to protracted alterations in mesolimbic CCK and concomitant alterations in fear, anxiety and motivation among enkephalin deficient mice. In any event, these data suggest that pharmacological management of anxiety disorders should be directed toward specific symptoms characterizing the psychological disorder.

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APPENDIX 1: EXPERIMENTAL ANOVA SPSS DATA TABLES

EXPERIMENT 1:

Analysis of variance of light-dark box behavior following exposure to rat odor

		Sum of Squares	DF	Mean Square	F	P
Latency To dark	BG	5197.400	10	519.740	2.122	.043
	WG	10530.600	43	244.898		
	Total	15728.000	53			
TRAN5MIN	BG	927.120	10	92.712	1.043	.425
	WG	3820.750	43	88.855		
	Total	4747.870	53			
TRAN10MI	BG	740.554	10	74.055	1.693	.114
	WG	1881.150	43	43.748		
	Total	2621.704	53			
LIGHT5	BG	67650.687	10	6765.069	2.149	.041
	WG	135368.350	43	3148.101		
	Total	203019.037	53			
LIGHT10	BG	62668.259	10	6266.826	2.251	.032
	WG	119723.000	43	2784.256		
	Total	182391.259	53			

Analysis of variance of CCK mRNA in the VTA, mPFC and subregions of the amygdala following rat odor exposure and light-dark box activity

VTA		Sum of Squares	DF	Mean Square	F	P
AREA	BG	19227605.636	10	1922760.564	3.076	.013
	WG	13753300.000	22	625150.000		
	Total	32980905.636	32			

		Sum of Squares	DF	Mean Square	F	P
OD	BG	.721	10	7.206E-02	1.342	.270
	WG	1.181	22	5.370E-02		
	Total	1.902	32			
		Sum of Squares	DF	Mean Square	F	P
ID	BG	40243645.323	10	4024364.532	2.832	.020
	WG	31266103.523	22	1421186.524		
	Total	71509748.846	32			

EXPERIMENT 1 (con'd):
mPFC - CCK mRNA

		Sum of Squares	DF	Mean Square	F	P
ODMEAN	BG	3.860	10	.386	2.364	.044
	WG	3.593	22	.163		
	Total	7.454	32			

BLA
AREAALLBLA

Source	Sum of Squares	DF	Mean Square	F	P
GROUP	6182417.672	10	618241.767	4.790	.001
Error	2581137.167	20	129056.858		
Total	209678600.000	31			

DIBLA

Source	Sum of Squares	DF	Mean Square	F	P
GROUP	100205053.887	10	10020505.38	4.175	.003
Error	47996680.500	20	2399834.025		
Total	993643537.000	31			

IDALLBLA

Source	Sum of Squares	DF	Mean Square	F	P.
GROUP	8.7E +07	10	8734606	4.247	.003
Error	4.1E +07	20	20568846		
Total	1.3E +08	30			

ODBLA

Source	Sum of Squares	DF	Mean Square	F	P
GROUP	5.097	10	.510	1.682	.154
Error	6.059	20	.303		
Total	161.196	31			

ODALLBLA

Source	Sum of Squares	DF	Mean Square	F	P
GROUP	5.212	10	.521	2.532	.037
Error	4.117	20	.206		
Total	155.739	31			

EXPERIMENT 1 (con'd):

MEA- CCK mRNA

AREA

Source	Sum of Squares	DF	Mean Square	F	P
GROUP	3805380.167	10	380538.017	.928	.528
Error	8611943.833	21	410092.563		
Total	193221452.000	32			

ODMEA

Source	Sum of Squares	DF	Mean Square	F	P
GROUP	5.688	10	.569	4.053	.003
Error	2.947	21	.140		
Total	195.170	32			

DIMEA

Source	Sum of Squares	DF	Mean Square	F	P
GROUP	63553265.875	10	6355326.587	1.462	.222
Error	91312396.000	21	4348209.333		
Total	1171848112.000	32			

EXPERIMENT 2 : Analysis of variance of peak startle amplitudes following exposure of mice to 2, 5 or 10 minutes of rat odor with repeated measures over days.

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
GROUP	128242.976	1	128242.976	30.47	.000
TIME	44356.490	2	22178.245	5.270	.011
GROUP * TIME	19447.498	2	9723.749	2.311	.118
Error	117832.154	28	4208.291		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	190.610	3	63.537 .024	.995	.071
DAY * GROUP	5130.219	3	1710.073	.633	.596
DAY * TIME	35043.617	6	5840.603	2.162	.055
DAY * GROUP TIME	43702.508	6	7283.751	2.697	.019
Error(DAY)	226896.478	84	2701.149		

EXPERIMENT 2 (con'd):Analysis of variance of peak startle amplitudes following exposure of mice to 10 minutes of rat odor with repeated measures over days.

		Sum of Squares	DF	Mean Square	F	P
IMM * group						
	BG	69620.958	9	7735.662	3.476	.005
	WG	68989.724	31	2225.475		
	Total	138610.681	40			
DAY2 * group						
	BG	35732.432	9	3970.270	2.453	.031
	WG	50183.913	31	1618.836		
	Total	85916.344	40			
DAY3 * group						
	BG	107334.801	9	11926.089	3.310	.006
	WG	111706.265	31	3603.428		
	Total	219041.066	40			
DAY7 * group						
	BG	25279.749	9	2808.861	1.832	.102
	WG	47537.468	31	1533.467		
	Total	72817.217	40			

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
GROUP	167242.830	9	18582.537	4.322	.001
Error	133300.326	31	4300.011		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P.
TIME	8030.421	3	2676.807	1.715	.169
TIME * GROUP	70725.110	27	2619.449	1.679	.036
Error(TIME)	145117.043	93	1560.398		

EXPERIMENT 3:

Analysis of variance of home-cage behaviors in response to TMT, BA or SAL exposure

		Sum of Squares	DF	Mean Square	F	P
#Cloth Contacts	BG	4591.16	2	2295.58	42.47	.000
	WG	2432.3	45	54.05		
	Total	7023.5	47			
Cloth Contacts (s)	BG	123006.5	2	61503.27	24.71	.000
	WG	111996.1	45	2488.80		
	Total	235002.7	47			
#Defensive Bury	BG	2310.87	2	115.438	15.117	.000
	WG	3439.438	45	76.432		
	Total	5750.313	47			
Defensive Bury (s)	BG	21381.54	2	10690.77	12.530	.000
	WG	38395.44	45	853.232		
	Total	59776.98	47			
#Digging	BG	579.87	2	289.94	1.762	.183
	WG	7403.94	45	164.53		
	Total	7983.8	47			
Digging(s)	BG	795.125	2	397.56	.329	.721
	WG	54339.87	45	1207.55		
	Total	55135.0	47			
#Freezing	BG	433.625	2	216.813	21.89	.000
	WG	445.687	45	9.90		
	Total	879.31	47			
Freezing(s)	BG	55008.5	2	27504.25	9.447	.000
	WG	131009.5	45	2911.322		
	Total	186018.0	47			
#Grooming	BG	12.667	2	6.33	1.955	.153
	WG	145.813	45	3.24		
	Total	158.479	47			
#Rears	BG	7910.292	2	3955.146	11.925	.000
	WG	14925.63	45	331.681		
	Total	22835.92	47			
#Stretch Attends	BG	234.542	2	117.271	9.3	.000
	WG	567.438	45	12.610		
	Total	801.979	47			

EXPERIMENT 3 (con'd):

Optical Density : CCK mRNA Basolateral Amygdala

	Sum of Squares	DF	Mean Square	F	P
Odor	1.671	2	.836	2.68	.09
Light-Dark Box	.031	1	.031	.101	.75
Odor * Light-Dark Box	2.251	2	1.126	3.62	.045
Residual	6.222	20	.311		

Mean Area : CCK mRNA Basolateral Amygdala

	Sum of Squares	DF	Mean Square	F	P
Odor	247798.4	2	123899.2	.970	.39
Light-Dark Box	27417.838	1	27417.838	.215	.65
Odor * Light-Dark Box	43710.29	2	21855.144	.171	.84
Residual	2555110.686	20	127755.534		

Integrated Density : CCK mRNA Basolateral Amygdala

	Sum of Squares	DF	Mean Square	F	P
Odor	20771987.8	2	10385993.9	3.14	.06
Light-Dark Box	704369.40	1	704369.40	.213	.65
Odor * Light-Dark Box	14012772.2	2	7006386.1	2.121	.14
Residual	66067803.5	20	3303390.18		

Optical Density : CCK mRNA mPFC

	Sum of Squares	DF	Mean Square	F	P
Odor	27.91	2	13.95	6.3	.008
Light-Dark Box	9.91	1	9.91	4.45	.048
Odor * Light-Dark Box	0.421	2	0.210	0.95	.91
Residual	6.222	19	.311		

Optical Density : VTA CCK mRNA

	Sum of Squares	DF	Mean Square	F	P
Odor	.543	2	.272	1.70	.207
Light-Dark Box	.331	1	.331	2.07	.165
Odor * Light-Dark Box	.331	2	.165	1.04	.373
Residual	3.191	20	.160		

EXPERIMENT 3 (con'd):
Mean Area : VTA CCK mRNA

	Sum of Squares	DF	Mean Square	F	P
Odor	1714371.06	2	857185.53	4.35	.027
Light-Dark Box	47165.81	1	47165.81	.240	.629
Odor * Light-Dark Box	362539.6	2	181269.8	.921	.414
Residual	3938483.50	20	196924.1		

Integrated Density : VTA CCK mRNA

	Sum of Squares	DF	Mean Square	F	P
Odor	19466038.75	2	9733019.37	4.42	.025
Light-Dark Box	3140371.4	1	3140371.4	1.43	.245
Odor * Light-Dark Box	1272544.8	2	636272.411	.290	.751
Residual	43824796.623	20	2191239.83		

EXPERIMENT 3 and 4:
Light-dark box behavior- Immediate test interval

		Sum of Squares	DF	Mean Square	F	P
FIVELITE	BG	35025.250	2	17512.625	6.566	.006
	WG	56011.250	21	2667.202		
	Total	91036.500	23			
TENLITE	BG	34246.333	2	17123.167	6.800	.005
	WG	52879.625	21	2518.077		
	Total	87125.958	23			
REENTER	BG	90.583	2	45.292	2.681	.092
	WG	354.750	21	16.893		
	Total	445.333	23			
FIVTRANS	BG	1246.583	2	623.292	3.868	.037
	WG	3384.375	21	161.161		
	Total	4630.958	23			
TENTRANS	BG	502.750	2	251.375	1.578	.230
	WG	3345.250	21	159.298		
	Total	3848.000	23			
TIMLIGHT	BG	140396.083	2	70198.042	7.631	.003
	WG	193189.250	21	9199.488		
	Total	333585.333	23			
TRANSIT	BG	3207.583	2	1603.792	2.664	.093
	WG	12644.375	21	602.113		
	Total	15851.958	23			

EXPERIMENT 4:
Analysis of variance of home-cage behaviors in response to TMT, BA or SAL exposure

		SUM OF SQUARES	DF	MEAN SQUARE	F	P
# CLOTH CONTACTS	BG	14976.573	2	7488.287	131.408	.000
	WG	9174.567	161	56.985		
	Total	24151.140	163			
CLOTH CONTACTS(s)	BG	598307.564	2	299153.782	152.252	.000
	WG	316342.876	161	1964.863		
	Total	914650.439	163			
#DEFENSIVE BURYING	BG	8773.735	2	4386.868	44.924	.000
	WG	15721.893	161	97.652		
	Total	24495.628	163			
DEFENSIVE BURYING (s)	BG	74262.472	2	37131.236	36.970	.000
	WG	161702.406	161	1004.363		
	Total	235964.878	163			
#FREEZING	BG	1604.591	2	802.295	75.425	.000
	WG	1712.549	161	10.637		
	Total	3317.140	163			
FREEZING (s)	BG	151848.647	2	75924.324	40.432	.000
	WG	302328.005	161	1877.814		
	Total	454176.652	163			
#DIGGING	BG	339.725	2	169.863	1.084	.341
	WG	25227.025	161	156.690		
	Total	25566.750	163			
DIGGING (s)	BG	10577.794	2	5288.897	4.473	.013
	WG	190346.566	161	1182.277		
	Total	200924.360	163			
#GROOMING	BG	59.029	2	29.515	7.347	.001
	WG	646.776	161	4.017		
	Total	705.805	163			
#REARS	BG	38951.530	2	19475.765	51.422	.000
	WG	60978.275	161	378.747		
	Total	99929.805	163			
#STRETCH	BG	964.224	2	482.112	32.633	.000
	WG2378.574		161	14.774		
	Total	3342.799	163			

EXPERIMENT 5:

Analysis of variance of peak startle of mature mice following a 10 minute exposure to TMT, BA or SAL

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
Odor	20682.0	2	10341.0	6.97	.008
Error	20772.06	14	1483.72		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	5821.41	3	1940.47	1.24	.305
DAY * ODOR	7477.89	6	1246.317	.800	.575
Error(DAY)	65430.77	42	1557.87		

Analysis of variance of peak startle of mature mice following a 2 minute exposure to TMT, BA or SAL

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
Odor	19289.43	2	9644.716	4.769	.043
Error	16180.22	8	2022.528		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	8256.78	3	2752.26	.860	.475
DAY * ODOR	13847.04	6	2307.84	.721	.637
Error(DAY)	76825.77	24	3201.07		

Juvenile mice: influence of a 10 minute odor exposure on acoustic startle

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
Odor	9486.43	2	4743.21	1.67	.228
Error	33942.06	12	2828.5		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	1453.914	3	484.6	.339	.797
DAY * ODOR	13511.7	6	2251.95	1.576	.182
Error(DAY)	65430.77	36	1428.76		

EXPERIMENT 5 (con'd):
Juvenile mice: influence of a 2 minute odor exposure on acoustic startle
Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
Odor	687.86	2	343.92	.264	.744
Error	11742.74	9	1304.75		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	652.03	3	217.344	.177	.911
DAY * ODOR	12089.24	6	2014.873	1.643	.174
Error(DAY)	76825.77	27	1225.97		

EXPERIMENT 6:
Analysis of variance of the influence of TMT, BA or SAL on ICSS from the dorsal aspects of the VTA.

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
ODOR	8824.837	2	4412.419	.584	.570
Error	113262.964	15	7550.864		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	38808.294	3	12936.098	5.111	.004
DAY * ODOR	20817.290	6	3469.548	1.371	.247
Error(DAY)	113890.988	45	2530.911		
FREQUENC	155058.690	6	25843.115	36.157	.000
FREQUENC * ODOR	30020.690	12	2501.724	3.500	.000
Error (FREQUENC)	64328.119	90	714.757		
DAY * FREQUENC	8406.929	18	467.052	1.842	.021
DAY * FREQUENC ODOR	4561.071	36	126.696	.500	.993
Error (D*F)	68457.929	270	253.548		

EXPERIMENT 7:
ANOVA of peak startle amplitudes following Boc-CCK-4 administration.

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DRUG	6031.768	2	3015.884	.590	.562
GROUP	19109.204	1	19109.204	3.741	.065
DRUG * GROUP	42754.068	2	21377.034	4.185	.028
Error	122590.237	24	5107.927		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	1650.965	3	550.322	.308	.820
DAY * DRUG	13031.658	6	2171.943	1.214	.309
DAY * GROUP	14967.132	3	4989.044	2.789	.047
DAY * DRUG GROUP	5846.760	6	974.460	.545	.772
Error(DAY)	128794.587	72	1788.814		

ANOVA of the startle latency following Boc-CCK-4 administration.

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DRUG	570.18	2	285.09	.234	.793
GROUP	5931.4	1	5931.4	4.86	.037
DRUG * GROUP	1079.32	2	539.7	.442	.028
Error	29278.87	24	1219.95		

EXPERIMENT 7 (con'd):
Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	984.819	3	328.273	.641	.591
DAY * DRUG	3621.617	6	603.603	1.178	.327
DAY * GROUP	580.873	3	193.624	.378	.769
DAY * DRUG GROUP	3543.665	6	590.611	1.153	.341
Error(DAY)	36878.226	72	512.198		

ANOVA of the peak startle amplitude following CCK-8S administration.
Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DRUG	34564.110	1	34564.110	4.191	.056
GROUP	172969.158	2	86484.579	10.486	.001
DRUG * GROUP	31914.546	2	15957.273	1.935	.173
Error	148453.877	18	8247.438		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	41330.177	3	13776.726	3.196	.031
DAY * DRUG	75397.770	3	25132.590	5.831	.002
DAY * GROUP	32567.803	6	5427.967	1.259	.292
DAY * DRUG GROUP	27399.072	6	4566.512	1.060	.398
Error(DAY)	232737.456	54	4309.953		

ANOVA of startle latency following CCK-8S administration.
Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P.
DRUG	2554.446	1	2554.446	.504	.487
GROUP	111841.675	2	55920.838	11.030	.001
DRUG * GROUP	24933.846	2	12466.923	2.459	.114
Error	91254.063	18	5069.670		

EXPERIMENT 7 (con'd):

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	9048.841	3	3016.280	2.864	.045
DAY * DRUG	3874.210	3	1291.403	1.226	.309
DAY * GROUP	19397.054	6	3232.842	3.070	.012
DAY * DRUG GROUP	12372.939	6	2062.157	1.958	.088
Error(DAY)	56865.734	54	1053.069		

ANOVA of startle duration following CCK-8S administration.

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DRUG	162.166	1	162.166	.117	.736
GROUP	23864.812	2	11932.406	8.644	.002
DRUG * GROUP	18732.207	2	9366.104	6.785	.006
Error	24846.483	18	1380.360		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	2291.784	3	763.928	1.192	.322
DAY * DRUG	868.383	3	289.461	.452	.717
DAY * GROUP	3796.540	6	632.757	.987	.443
DAY * DRUG GROUP	9983.609	6	1663.935	2.595	.028
Error(DAY)	34619.385	54	641.100		

EXPERIMENT 8: Analysis of variance with repeated measures for changes in ICSS performance following intraventricular CCK-8S and DALA administration.

Tests of Between-Subjects Effects

Source Type III	Sum of Squares	DF	Mean Square	F	P
DRUG1	104231.359	1	104231.359	6.852	.014
DRUG2	107580.296	1	107580.296	7.072	.013
DRUG3	40801.291	1	40801.291	2.682	.113
DRUG1 * DRUG2	21291.174	1	21291.174	1.400	.247
DRUG1 * DRUG3	54448.642	1	54448.642	3.579	.069
DRUG2 * DRUG3	21664.162	1	21664.162	1.424	.243
DRUG1 * DRUG2 * DRUG3	136836.950	1	136836.950	8.995	.006
Error	425946.385	28	15212.371		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DAY	410818.748	8	51352.343	13.11	.000
DAY * DRUG1	242213.001	8	30276.625	7.731	.000
DAY * DRUG2	62218.994	8	7777.374	1.986	.049
DAY * DRUG3	6864.777	8	858.097	.219	.987
DAY * DRUG1 * DRUG2	43830.294	8	5478.787	1.399	.198
DAY * DRUG1 * DRUG3	43348.429	8	5418.554	1.384	.205
DAY * DRUG2 * DRUG3	19407.661	8	2425.958	.619	.761
DAY * DRUG1 * DRUG2 * DRUG3	51917.953	8	6489.744	1.657	.110
Error(DAY)	877288.508	224	3916.467		
FREQ	487180.638	6	81196.773	61.90	.000
FREQ * DRUG1	41416.557	6	6902.759	5.263	.000
FREQ * DRUG2	21527.298	6	3587.883	2.735	.015
FREQ * DRUG3	2400.210	6	400.035	.305	.934
FREQ * DRUG1 * DRUG2	25045.127	6	4174.188	3.182	.006
FREQ * DRUG1 * DRUG3	2844.866	6	474.144	.361	.902

EXPERIMENT 8 (con'd):

FREQ * DRUG2 DRUG3	4656.676	6	776.113	.592	.737
FREQ * DRUG1 DRUG2 DRUG3	4209.690	6	701.615	.535	.781
Error(FREQ)	220356.457	168	1311.646		
DAY * FREQ	25763.892	48	536.748	1.462	.023
DAY * FREQ DRUG1	20612.600	48	429.429	1.169	.202
DAY * FREQ DRUG2	14670.103	48	305.627	.832	.786
DAY * FREQ DRUG3	21413.118	48	446.107	1.215	.152
DAY FREQ DRUG1 DRUG2	20067.167	48	418.066	1.138	.242
DAY * FREQ DRUG1 DRUG3	27601.616	48	575.034	1.566	.009
DAY * FREQ DRUG2 DRUG3	23561.889	48	490.873	1.337	.064
DAY FREQ DRUG1 DRUG2 DRUG3	18726.837	48	390.142	1.062	.360
Error(DAY*FREQ)	493532.378	1344	367.212		

EXPERIMENT 9: Analysis of variance with repeated measures for changes in locomotor activity following intraventricular CCK-8S and DALA administration.

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
DRUG1	522446.681	1	522446.681	5.402	.029
DRUG2	2308624.822	1	2308624.822	23.872	.000
DRUG3	1102.996	1	1102.996	.011	.916
DRUG1 * DRUG2	214111.494	1	214111.494	2.214	.150
DRUG1 * DRUG3	275871.511	1	275871.511	2.853	.104
DRUG2 * DRUG3	1662.438	1	1662.438	.017	.897
DRUG1 * DRUG2 DRUG3	282145.760	1	282145.760	2.917	.101
Error	2320995.906	24	96708.163		

Tests of Within-Subjects Effects

Source	Sum of Squares	df	Mean Square	F	Sig.
TIME	12184150.671	8	1523018.834	20.805	.000
TIME * DRUG1	1929526.975	8	241190.872	3.295	.002
TIME * DRUG2	13266578.115	8	1658322.264	22.653	.000
TIME * DRUG3	58343.933	8	7292.992	.100	.999
DRUG1 DRUG2 TIME * DRUG1	1600965.880	8	200120.735	2.734	.007
DRUG3 TIME * DRUG2	2795857.627	8	349482.203	4.774	.000
DRUG3 TIME DRUG1 DRUG2	60455.231	8	7556.904	.103	.999
DRUG3	2806462.350	8	350807.794	4.792	.000
Error(TIME)	14055164.201	192	73203.980		

EXPERIMENT 9 (con'd):

Analysis of variance with repeated measures for changes in rearing behavior following intraventricular CCK-8S and DALA administration.

Tests of Between-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	Sig.
DRUG1	141134.366	1	141134.366	7.376	.012
DRUG2	162996.990	1	162996.990	8.519	.008
DRUG3	13515.266	1	13515.266	.706	.409
DRUG1 * DRUG2	70897.082	1	70897.082	3.705	.066
DRUG1 * DRUG3	7813.984	1	7813.984	.408	.529
DRUG2 * DRUG3	5366.123	1	5366.123	.280	.601
DRUG1 * DRUG2 DRUG3	5697.465	1	5697.465	.298	.590
Error	459214.781	24	19133.949		

Tests of Within-Subjects Effects

Source	Sum of Squares	DF	Mean Square	F	P
TIME	1267318.626	8	158414.828	14.14	.000
TIME * DRUG1	116573.309	8	14571.664	1.301	.245
TIME * DRUG2	1508688.563	8	188586.070	16.83	.000
TIME * DRUG3	71640.280	8	8955.035	.799	.604
TIME * DRUG1 DRUG2	28616.352	8	3577.044	.319	.958
TIME * DRUG1 DRUG3	140349.692	8	17543.711	1.566	.137
TIME * DRUG2 DRUG3	30682.478	8	3835.310	.342	.948
TIME * DRUG1 DRUG2 DRUG3	87542.867	8	10942.858	.977	.455
Error(TIME)	2150735.178	192	11201.746		