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Stressor Exposure and Intraventricular Cholecystokinin (CCK-8) Administration in the Light Dark Box Model of Anxiety in CD-1 Mice: Possible Cross-Sensitization

by

Glenda Mac Neil, B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Master of Science
Specialization in Neuroscience

Department of Psychology

Carleton University
Ottawa, Ontario
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May 9, 1996
ABSTRACT

The behavioural consequences of stressor exposure and intraventricular administration of CCK-8S were evaluated in the light-dark paradigm in CD-1 mice. Exposure to 15, 30 or 60 uncontrollable footshocks significantly augmented anxiety, while only 30 minutes of restraint provoked significant behavioural alterations. Fifteen or 60 minutes of restraint produced slight, but non-significant increases in anxiety. Anxiety levels were also affected by experiential factors, such as a history of surgical stress. The intraventricular administration of 5 ng CCK-8S was ineffective at provoking anxiety on the initial test day and upon re-exposure, one week later, while 50 ng CCK-8S produced significant behavioural alterations on both occasions. Interestingly, the 25 ng dose of CCK-8S augmented anxiety upon re-exposure, but did not provoke anxiety following initial challenge. Moreover, behavioural cross-sensitization between 30 minutes of restraint stress and the 5 ng dose of CCK-8S resulted in heightened anxiogenic reactivity. Cross-sensitization was not demonstrated if mice had been exposed to uncontrollable footshock prior to CCK-8S or if CCK-8S administration preceded stressor exposure. Cholecystokinin and stressor induced anxiety likely follow from an interaction of multiple neurotransmitter/neuropeptide systems and may be exacerbated by the induction of sensitization and cross-sensitization. Such neurochemical alterations may influence sensitivity to future stressors and vulnerability to stressor-related disorders.
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INTRODUCTION

Cholecystokinin (CCK), a gastrointestinal hormone, was initially associated with gall bladder contraction and pancreatic enzyme secretion (Beinfeld, Mever, Eskay, Jensen & Brownstein, 1981). It was subsequently demonstrated that this gastrin-like peptide is also discernible in the central nervous system (CNS) (Saito, Sankaran, Goldfine & Williams, 1980; Vanderhaeghen et al., 1981). Indeed, CCK, isolated as a 33 amino acid peptide chain, appears in both the brain and periphery in various molecular forms (Innis and Snyder, 1980). The sulfated octapeptide, CCK-8, is the predominant version of CCK in the CNS (Albus, 1988) and central, as well as peripheral CCK receptors, have a greater affinity for CCK-8 than for other forms of the peptide (Goltermann, 1985).

Central CCK availability is considerable. The cerebral cortex is reported to contain a substantial proportion of total brain CCK, with a high concentration in the cingulate, pyriform and entorhinal cortices (Beinfeld et al., 1981). Numerous studies employing radioimmunoassay, immunohistochemistry and autoradiography have also detected CCK in several additional brain sites. For example, high levels of the peptide have been detected in subnuclei of the amygdala (Crawley, 1985a), the CA1 layer of the hippocampus (Lanaud et al., 1989), numerous divisions of the paraventricular nucleus of the hypothalamus (Micevych, Park, Akesson & Elde, 1987), the periaqueductal grey (Beinfeld et al., 1981, Larsson & Rehfeld, 1979, Tang & Man, 1989, Vanderhaeghen et al., 1981), as well as the septum, olfactory tubercle, thalamus, dorsal and median raphe, the substantia nigra and the ventral tegmental area, (Albus, 1988, Crawley, 1985a, Fallon & Serogy, 1985, Hokfelt et al., 1991). Much of the data pertaining to central CCK
availability has been accumulated employing infrahuman subjects. Accordingly, it should be considered that differences are not only evident in the distribution of CCK and CCK receptor density among infrahuman subjects, but also between humans and infrahumans (Dietl & Palacios, 1989).

The subtypes of central CCK receptors identified to date are CCK\textsubscript{\textalpha} and CCK\textsubscript{\textbeta}. The CCK\textsubscript{\textalpha} receptor was initially identified in the periphery and subsequently localized in a few discrete areas of the brain (Moran, Robinson, Goldrich & McHugh, 1986). This receptor has a high affinity for the sulfated octapeptide, CCK-8, while the CCK\textsubscript{\textbeta} receptor, the prominent CCK receptor in the brain, has an affinity for CCK-8-sulfated, CCK-8-unsulfated, CCK-4, and pentagastrin (Saito et al., 1980). Electrophysiological experiments have revealed that the predominant action of the peptide is excitatory (Boden & Woodruff, 1994). Although the neuronal consequences of CCK administration have been well documented, the functional significance of CCK release in the CNS is poorly understood. Despite such difficulties, it has been argued that inter-regional variability in the concentration of the peptide may indicate that CCK participates in the modulation of behaviour associated with specific brain areas. In particular, dense CCK labeling within the periaqueductal grey has implicated CCK in analgesia (Lanaud et al., 1989). CCK in the nigrostriatal system suggests a role for the peptide in motoric output (Crawley, Hays, Paul & Goodwin, 1981, Harro & Vasar, 1991a, b), and hypothalamic CCK points to a potential influence of CCK in satiety (Crawley, 1985b, Dourish, Ruckert, Tattersall & Iversen, 1989). In fact, central administration of CCK has been reported to produce mild analgesia.
(Jurna & Zetler, 1981), to potentiate DA induced hyperlocomotion and to inhibit feeding (Crawley, 1985b)

In addition to the induction of behavioural alterations following CCK administration, disturbances of this peptidergic system have been associated with behavioural pathology. For example, considerable attention has been devoted to an analysis of alterations of CCK activity in schizophrenia (Albus, 1988, Crawley, 1991, Rasmussen, Stockton, Czachura & Howbert, 1991, Schiantarelli, 1993) and the widespread distribution of CCK and CCK receptors in limbic areas has raised the possibility that CCK may also be implicated in emotional reactivity and anxiety, in particular (Woodruff et al., 1991). The evidence in support of a CCK involvement in anxiety has increased considerably following the discovery that CCK-induced excitation of hippocampal neurons was suppressed by benzodiazepine administration (Bradwejn & de Montigny, 1984). Moreover, peripheral or intracerebral administration of CCK agonists induces anxiogenic behaviour in animals (Harro, Pold & Vasar, 1990, Harro & Vasar, 1991b, Singh, Field, Hughes, Vass & Woodruff, 1991) and systemic CCK-4 administration among healthy volunteers provokes panic attacks reminiscent of those evident in panic patients (Bradwejn & Koszycki, 1994). In contrast, selective CCK antagonists antagonize CCK induced anxiety and produce an anxiolytic profile in animals (Hendrie, Neill, Shepherd & Dourish, 1993, Ravard, Dourish & Iversen, 1990, Singh, Lewis, Field, Hughes & Woodruff, 1991), as well as in panic patients (Bradwejn et al., 1994).
Paralleling the behavioural disturbances evident following CCK administration, exposure to uncontrollable stressors also produces behavioural alterations in animal models of pathology (Zacharko & Anisman, 1991) including shuttle-escape disturbances (Maier & Seligman, 1976), water maze deficits (Prince & Anisman, 1984), disruptions of appetitively motivated behaviour (Rosellini, 1978), increased latencies in tests of analgesia (e.g., tail flick) (Mah, Suissa & Anisman, 1980, Maier, Drugan & Grau, 1982), exploratory changes (Bruto & Anisman, 1983), response perseveration (Anisman, Hahn, Hoffman & Zacharko, 1985) and reduced responding for intracranial self-stimulation (ICSS) (Zacharko, Bowers, Kokkinidis & Anisman, 1983) (for reviews see Anisman, Zalcman, Shanks & Zacharko, 1991; Zacharko & Anisman, 1984). The induction, magnitude and time course of these behavioural impairments have been associated with variations in several neurotransmitter systems. Although the nature of the relationship between aversive life events, neurochemical alterations and pathology remains to be clarified, it has been postulated that uncontrollable stressors may contribute to the expression and/or exacerbation of a variety of physical or psychological illnesses, including depression and anxiety (Weiss & Simpson, 1984; Zacharko & Anisman, 1991).

The ensuing sections of this review examine the experimental evidence supporting a role for central CCK-8 activity in the provocation of anxiety and consider the argument that stressors may contribute to this pathology. Accordingly, the discussion will be extended to a consideration of the potential neuromodulatory influence of CCK-8 on central neurotransmitters implicated in anxiety, including gamma-aminobutyric acid (GABA), norepinephrine (NE), dopamine (DA) and serotonin (5-HT). In addition, the
contribution of stressors to behavioural change and the specific neurochemical alterations induced by aversive events will be discussed. Finally, in view of the observation that both CCK administration and stressors may result in similar behavioural and/or neurochemical profiles, behavioural cross-sensitization between stressors and CCK in the promotion and/or exacerbation of pathology will also be considered.

CENTRAL NEUROCHEMICAL ACTIVITY AND ANXIETY

**Gamma-aminobutyric acid (GABA) and the Benzodiazepines**

Chronic benzodiazepine administration, the treatment of choice for anxiety disorders, facilitates GABA transmission (Haefley, 1992, Matsumoto, 1989). It is not surprising, therefore, that an analysis of the neurochemical correlates underlying the expression of anxiety has focused on the GABA$_A$-benzodiazepine receptor complex. Benzodiazepine receptors are located primarily in the frontal cortex, limbic areas, the substantia nigra, superior colliculus and the ventral nucleus of the lateral lemniscus (Ferrarese et al, 1993). It will be recalled that many of these central areas also contain appreciable concentrations of CCK (Lanaud et al, 1989, Larsson & Rehfeld, 1979). Diazepam, chlordiazepoxide or flurazepam binding to benzodiazepine receptors produce anxiolytic effects in clinical populations and many animal models of anxiety (Assie, Chopin, Palmier & Briley, 1991, Cole & Rodgers, 1993). Conversely, the inverse agonists including β-CCM, β-CCE and FG 7142 decrease the inhibitory effects of GABA and provoke anxiety (Belzung, Missetlin, Vogel, Dodd & Chapouthier 1987, Sulcova, Krsiak, & Donat 1992). The benzodiazepine antagonists (e.g., RO 15-1788, flumazenil) prevent the
actions of agonists and the inverse agonists, but possess little intrinsic activity (Shephard, 1986) It is intriguing that the novel β-carboline, ZK91296, ordinarily identified as an anxiogenic agent, produces anxiolytic effects in some animal models of anxiety (e.g., the social interaction paradigm), while RO 15-1788 has produced anxiogenic effects in other models (e.g., Vogel punished drinking task) (File & Baldwin, 1987; File & Pellow, 1986). The source(s) of such variability remain(s) to be determined.

The identification of central benzodiazepine receptors has prompted attempts to identify endogenous ligands which may promote anxiety. One potential ligand which has been identified to date is diazepam binding inhibitor (DBI) (Cooper, Bloom & Roth, 1991). Although the precise function of DBI is presently unknown, some investigations suggest that DBI may be involved in mediating the response to stressors by increasing the release of corticotrophin-releasing factor (Salzman, Miyawaki, Le Bars & Kerrihard, 1993), or may exacerbate anxiety through downregulation of GABA transmission (Ferrarese et al., 1993). It is interesting to consider that anxiety disorders may follow, at least in part, from an imbalance between endogenous anxiogenic and anxiolytic agents (Salzman et al., 1993).

In addition to a central GABA influence, the clinical efficacy of benzodiazepines in the treatment of anxiety may be associated with the inhibition of adenosine reuptake, the attenuation of 5-HT activity, a reduction of NE turnover, and/or antagonism of CCK induced neuronal excitation (Polc, 1991). The suggestion that antagonism of CCK might be involved in the anxiolytic effects of benzodiazepines stems from the research of Bradwejn and de Montigny (1984). These investigators demonstrated that low doses of
benzodiazepines, applied microiontophoretically to rat hippocampal pyramidal neurons, antagonized CCK-induced excitation, but did not block the excitatory effects of acetylcholine, met-enkephalin, aspartate or glutamate. In addition, a two week schedule of diazepam or flurazepam administration markedly reduced the excitatory effects of microiontophoretically applied CCK on rat CA3 hippocampal pyramidal neurons (Bouthillier & de Montigny, 1988). Moreover, 24 hours following the cessation of chronic diazepam treatment, CCK-8 receptors were significantly increased in the frontal cortex and hippocampus in rats (Harro, Lang & Vasar, 1990). It was suggested that such increased CCK-8 receptor density may parallel the rebound anxiety evident when benzodiazepines are discontinued in clinical applications. Finally, a dose-dependent increase in the number of CCK-8 receptor sites was also demonstrated in the rat frontal cortex following acute administration of the anxiogenic, FG 7142 (Harro, Kiivet, Lang & Vasar, 1990).

The functional antagonism between CCK-8 and the benzodiazepines is further supported by the finding that proglimide, a CCK-8 antagonist, blocked the behavioural effects of the anxiogenic β-carboline derivative, DMCM, which presumably decreases GABA turnover (Harro et al., 1990a). Moreover, Cl-988, the selective and potent CCK₈ receptor antagonist (Hughes et al., 1990), dose-dependently antagonized both the anxiogenic and proconvulsant effects in mice tested in the light-dark box following diazepam withdrawal (Singh, Field, Vass, Hughes & Woodruff, 1992). Interestingly, in a study which separated rats into ‘anxious’ and ‘non-anxious’ conditions based on their behaviour in the elevated plus maze, Harro and coworkers (1990a) noted that ‘anxious’
rats had significantly fewer benzodiazepine receptors and significantly more CCK receptors in the frontal cortex than non-anxious animals. Finally, the benzodiazepine antagonist, flumazenil, blocked the behavioural effects of CCK agonists and antagonists in rats tested in the elevated plus maze. These findings allude to a more complex interaction between benzodiazepine and CCK receptors in the regulation of anxiety states than was originally postulated (Chopin & Briley, 1993).

In summary, benzodiazepines enhance GABA neurotransmission and may modulate CCK activity and/or other neurotransmitter systems in producing an anxiolytic effect. Physiological, as well as pharmacological studies, however, strongly suggest that multiple neurochemical systems, including NE, DA and 5-HT, are also involved in the pathophysiology of anxiety (Charney et al., 1990b).

**Norepinephrine**

The locus coeruleus (LC), which produces approximately 70% of the NE identified in the CNS (Ballenger, Post, Jimerson, Lake & Zuckerman, 1984; Svensson, 1987) has diverse efferent projections to numerous CNS sites including the cerebral cortex, the limbic system and the olfactory bulbs (Cooper et al., 1991). It might be noted parenthetically that high concentrations of CCK have also been reported in the cerebral cortex and the limbic system (Beinfeld et al., 1981; Crawley, 1985a, Vanderhaeghen et al., 1981). One of the functions of NE is to increase the signal-to-noise ratio of post-synaptic neurons or 'bias target cells to respond preferentially to their strongest input' (Aston-Jones, Chiang & Alexinsky, 1991). Likewise, numerous reports indicate that the LC-NE system participates in the promotion of arousal and vigilance (Aston-Jones & Bloom,
1981, Foote, Berriuge, Adams & Pineda, 1991) and may influence the processing of external stimuli such that attention to irrelevant stimuli is suppressed and attention to immediate and relevant stimuli is increased (Aston-Jones et al., 1991). In effect, activation of the dorsal NE bundle may be adaptive, perhaps preparing an organism to respond to salient environmental stimuli (Charney et al., 1990b, Johnston, 1991) and mobilizing an organism for fight or flight (Svensson, 1987).

In addition to a potential role of NE neurons in attentional processing, it has been suggested that central NE activation may have a significant influence on the sleep-wake cycle and learning and memory and may participate in various disorders, including anxiety and panic (Aston-Jones et al., 1991). Some investigators have argued that the expression of anxiety is occasioned by NE overactivity, while an attenuation of NE hyperactivity favours anxiety reduction (Johnston, 1991; Nutt & Glue, 1991). The proposal that the LC-NE system may contribute to anxiety induction was prompted by several lines of evidence. For example, electrical stimulation of the LC or intravenous administration of the $\alpha_2$-NE antagonists, yohimbine or piperoxane, provoked fear behaviours in monkeys similar to those exhibited following exposure to a threatening situation (e.g., scratching and mouth movements) (Redmond & Huang, 1979). Yohimbine, which increases LC unit activity and NE turnover by blocking the $\alpha_2$-NE autoreceptor, likewise promoted anxiety following systemic administration in humans (Charney, Heninger, & Redmond, 1983, Nutt, 1989). The induction of behaviour reminiscent of anxiety following administration of NE antagonists has also been detected in various animal models of anxiety employing rats and mice (Blanchard, Taukulis, Rodgers, Magee & Blanchard, 1993, Pellow, Chopin & File,
1985, Venault, Jacquot, Save, Sara & Chapouthier, 1993) (but not all, see Baldwin, Johnston, & File, 1989) It remains to be determined whether the anxiogenic effects of yohimbine are peculiar to the \( \alpha_2 \)-NE site, however (Johnston, 1991), since yohimbine apparently has indirect effects on cholinergic, serotonergic and dopaminergic systems as well (Charney et al., 1983, Johnston & File, 1989, Uhde & Tancer, 1989).

In contrast to the anxiogenic effects induced by \( \alpha_2 \)-NE antagonists, bilateral LC lesions in monkeys decreased anxious behaviour in response to threatening stimuli and the administration of the \( \alpha_2 \)-NE agonist, clonidine, attenuated the fearful behaviour induced by electrical stimulation of the LC (Redmond & Huang, 1979). Clonidine also antagonized yohimbine induced anxiety and attenuated yohimbine induced increases in plasma 3-methoxy-4-hydroxy-phenylethanolamine (MHPG) and blood pressure in healthy volunteer subjects (Charney, et al., 1983) and in patients with panic disorder (Nutt, 1989). In another report, however, the short-term anxiolytic effects of clonidine in patients with panic disorder did not persist with long-term prophylactic administration (Uhde et al., 1989). Further evidence supporting a role for NE in the manifestation of anxiety is derived from the correlation between subjective reports of anxiety in humans and peripheral measures of central NE activity. Plasma and urinary NE and/or MHPG levels appear to be elevated in patients with generalized anxiety and panic disorder relative to control subjects (Ballanger et al., 1984; Johnston, 1991). The precise relationship between peripheral measures of amine metabolites and the CNS is not clear, however and the significance of these data to central mechanisms has been challenged (see Kopin, 1992).
In view of the demonstrated inhibitory influence of GABA on LC activity and the observation that increased GABA activity or benzodiazepine administration attenuates LC activity (Charney & Heninger, 1986), it is likely that the anxiolytic effects of benzodiazepines are linked to an NE influence (Yang, Luo & Zhou, 1988). Indeed, diazepam, for example, blocks the behavioural effects of LC stimulation and reduces stressor induced increases in NE turnover in LC projection areas (Ballenger et al., 1984, Johnston, 1991). In contrast, the anxiogenic effects of yohimbine in rats in the elevated plus maze are not reversed by chloridiazepoxide or by the benzodiazepine antagonist, Ro 15-1788 (File, 1987). Moreover, the anxiogenic effects of idazoxan are not blocked by Ro 15-1788 in the light-dark box or in a conditioned conflict situation (Venault et al., 1993). Accordingly, benzodiazepines affect NE turnover, but there is little direct evidence attributing the anxiolytic profile of benzodiazepines to reduced NE turnover per se (Johnston, 1991). In addition, the anxiogenic consequences of α2-NE antagonists in animal tests of anxiety can not be accounted for entirely by actions at the benzodiazepine receptor. It is of note, however, that Harro et al. (1992) recently demonstrated that denervation of NE input from the LC to projection areas following DSP4 lesions resulted in an upregulation of CCK receptors in the hippocampus and frontal cortex (Harro, Jossan & Oreland, 1992). Consequently, the possibility that CCK-NE interactions may play a role in anxiety deserves further scrutiny.

Despite the evidence favouring NE involvement in anxiety, recent clinical investigations present data inconsistent with an NE hypothesis of anxiety (Pohl, Rainey, Ortiz & Yeragani, 1987). For example, Kaitin et al. (1986) reported that, in contrast to
previous data in monkeys (Redmond & Huang, 1979). LC stimulation in an epileptic patient produced relaxation. The results of a subsequent report involving electrical stimulation of the LC in three patients provided comparable data (Libet & Gleason, 1994). Electrical stimulation failed to provoke subjective reports of anxiety or any evidence of discomfort in any of the subjects tested. It should be underscored, nevertheless, that these data were derived from epileptic patients and, in one case, an individual with cerebral palsy who was uncommunicative (Libet & Gleason, 1994). Although these data are interesting, it is questionable whether conclusions pertaining to the neural mechanisms associated with NE activity and anxiety can be gleaned from individuals with a challenged CNS.

Some additional data which contradict the notion that LC activation potentiates anxiety are derived from Wiess et al. (1994) who proposed that increased activity of the LC may actually exert an anti-anxiety influence. Such a proposal is based on the findings that bilateral LC infusions of desipramine, which inhibits LC unit activity, increased anxious behaviour in an open field drink test (i.e., decreased drinking time and increased immobility), while infusions of idazoxan and substance P, which increases LC activity, decreased anxious behaviour in the same paradigm. On the basis of these data, the authors challenge the theory that activation of the LC-NE system promotes anxiety and suggest the LC may have a counter-balancing, restorative function. Although these conclusions are intriguing, it should be considered that these data may be paradigm specific. In effect, NE alterations may be sufficient to promote an anxiolytic influence in this paradigm, but may not be adequate to modify behaviour in other models of anxiety.
Taken together, there is evidence to suggest NE involvement in anxiety, but available data indicate that the manifestation of anxiety is not a consequence of a simple alteration of LC-NE function. In some cases, NE alterations may be adequate to promote anxiety, however, anxiogenesis may also involve a failure of central mechanisms that interface with NE (e.g., GABA) (Nutt, 1989), or a disturbance in other neurotransmitter or putative neurotransmitter (i.e., neuropeptide) systems (Dubovsky, 1993). Moreover, it is conceivable that the LC-NE system plays a role in regulating attention to salient environmental stimuli and consequently, contributes to ensuing cascading neurochemical events and the eventual behavioural expression of anxiety.

**Dopamine**

Alterations of central DA activity have been associated with variations in locomotor activity and exploration (Carlsson, 1993), psychostimulant drug self-administration (Roberts, Zis & Fibiger, 1975, Vaccarino, 1994), and the regulation of feeding (Radhakishun, van Ree & Westernik, 1988), among other behaviours. Clinically, altered DA turnover has been linked to schizophrenia (Losonczy, Davidson & Davis, 1987), Parkinson's disease (Hornykiewicz & Kish, 1986) and the induction of anxiety (Biggio et al., 1990; Tam & Roth, 1985). The ventral tegmental area (VTA), A10 region, is the point of origin of the DA containing mesocortical and mesolimbic systems which have typically been associated with the mediation of reward and motivation (Fibiger & Phillips, 1988, LeMoal & Simon, 1991). The promotion of DA activity within these systems following exposure to aversive events has been related to anhedonic and anxiogenic effects in various animal models of psychological pathology (e.g., ICSS
paradigm, exploratory tests, elevated plus maze and light-dark box) (Roth, Tam, Ida, Yang & Deutch, 1988, Zacharko & Anisman, 1991). Accordingly, it is argued that an attenuation of DA activity may be linked with anxiolytic effects (Biggio et al., 1990, Simon, Panissaud & Costentin, 1993).

The proposed involvement of DA in anxiety is supported by the observation that benzodiazepines prevent stressor provoked increases in DA metabolism in the mesoprefrontal cortex and in the nucleus accumbens. For example, Laveille et al. (1978) and Kramer and Petty (1989) demonstrated that acute administration of diazepam or chlordiazepoxide prevented the increase in the DOPAC/DA ratio ordinarily evident in the frontal cortex of rats following stressor exposure. Likewise, chronic administration of the benzodiazepine, midazolam decreased extracellular DA concentrations in the nucleus accumbens by 50% (Finlay, Damsma & Fibiger, 1992).

In addition to the observation that benzodiazepines reverse stressor induced DA turnover, the anxiogenic β-carboline, FG 7142, like uncontrollable footshock, promoted a selective activation of DA turnover in the frontal cortex (Tam & Roth, 1985). Similar observations were reported recently where FG 7142 enhanced the release of cortical DA by at least 50% (Moghaddam, Roth & Bunney, 1990). The inverse agonists, β-CCE and FG 7142 also produced dose-related increases in DA release and metabolism in the nucleus accumbens (McCullough & Salamone, 1992). It is of note that the benzodiazepine antagonist, Ro 15-1788, blocked the ameliorating effects of several anxiolytics (i.e., prevention of stress-induced DA turnover), and prevented the activation of DA neurons associated with the administration of anxiogenic inverse agonists (Biggio et al., 1990).
Taken together, these data provide evidence consistent with the involvement of mesocorticolimbic DA and benzodiazepine receptors in the modulation of anxiety.

At least 40% of DA neurons in the VTA of the rat also contain CCK (Hokfelt et al., 1980). Moreover, DA and CCK are co-localized in the prefrontal cortex, nucleus accumbens, medial septum, olfactory tubercle and the central nucleus of the amygdala (Hokfelt et al., 1985, Bunney, 1987). Consistent with these observations, chronic treatment with the DA receptor antagonist, haloperidol, significantly increased CCK receptor density in the cerebral cortex and nucleus accumbens (Chang, Lotti, Martin & Chen, 1983), while acute or chronic administration of CCK significantly increased the number of D₂ receptor binding sites in the cortex, nucleus accumbens and the olfactory tubercle (Crawley, 1991).

Microiontophoretic application of CCK-8 onto cell bodies of the VTA or substantia nigra elicits neuronal excitation (Crawley. 1991, Freeman & Bunney, 1987). Likewise, application of CCK in the prefrontal cortex also increased the spontaneous activity of DA-sensitive neurons (Chiodo & Bunney, 1983). In the nucleus accumbens, however, CCK was found to excite only those cells in the dorsal medial aspect of the nucleus (Hokfelt et al., 1980). Paradoxically, when CCK is administered in conjunction with DA or a DA agonist, CCK potentiates the inhibitory effects of DA on tegmental neurons (Freeman & Bunney, 1987, Hommer & Skirboll, 1983) and blocks the inhibitory effects of DA in the nucleus accumbens (Yim & Mogenson, 1991).

The mesolimbic CCK/DA fibers originating in the VTA provide distinct projections to the medial, posterior region of the nucleus accumbens (Hurd et al., 1992),
while innervation from the A10 area to the anterior accumbens appears to be a non-CCK containing pathway. Therefore, any neurochemical detection of CCK in this region is likely derived from cortical areas (Crawley, 1992, Hokfelt et al., 1985) Cholecystokinin potentiates DA release, DA-mediated hyperlocomotion and amphetamine induced hyperlocomotion following posterior nucleus accumbens administration (Vaccarino & Rankin, 1989, Vaccarino, 1994) Results of studies employing specific receptor subtype antagonists demonstrate the CCK\textsubscript{\lambda} receptor mediating these effects (Crawley, 1992, 1994) Conversely, CCK has been shown to inhibit DA release, DA-mediated behaviours and amphetamine induced hyperlocomotion in the anterior accumbens (Vaccarino & Rankin, 1989) and these modulatory effects appear to be a function of CCK\textsubscript{\beta} receptor mechanisms (Crawley, 1992, 1994)

Cholecystokinin-DA interactions have also been demonstrated in a number of laboratories employing CCK\textsubscript{\beta} receptor antagonists In particular, acute administration of the CCK\textsubscript{\beta} receptor antagonists LY262691, LY262684, LY191009 or LY242040, but not CCK\textsubscript{\lambda} antagonists, decreased the number of spontaneously active DA neurons in the VTA of the rat (Rasmussen et al., 1991; Rasmussen, Czachura, Stockton & Howbert, 1993) In addition, microinjection of LY262691 into the nucleus accumbens or medial prefrontal cortex led to a significant decrease in the number of spontaneously active A10 DA cells, but was without effect on A9 activity. Therefore, it was suggested that the effects of LY262691 on A10 cells are mediated, at least in part, by the antagonism of CCK\textsubscript{\beta} receptors in the nucleus accumbens and prefrontal cortex (Rasmussen, Howbert & Stockton, 1993) Clearly, additional investigations are necessary to determine the exact
mechanisms required for CCK antagonists to inhibit A10 activity. It is interesting to speculate, however, that CCK\textsubscript{A} antagonists may eliminate the excitatory effects of CCK, which contribute to the activation of DA neurons during stress, and thereby reduce anxiety associated with exposure to uncontrollable aversive events (Crawley, 1991; Rasmussen et al., 1993).

In view of the extensive colocalization of CCK and DA and the anxiogenic effects associated with increased activity in the mesocorticolimbic system following the administration of CCK or CCK agonists, a role for CCK-DA interactions in the induction of anxiety is likely. Moreover, a benzodiazepine/GABA receptor link in mediating DA turnover in response to stressors or β-carboline administration deserves consideration. Taken together, these data demonstrate at least a modulatory role for DA in the promotion of anxiety.

**Serotonin**

Serotonin has been implicated in temperature regulation, sleep, learning and memory, pain perception, feeding, sexual behaviour, and aggressive or impulsive behaviour (Eison, 1990). In addition, aberrant 5-HT neurotransmission has been associated with eating disorders (Advokat & Kutlesic, 1995, Jimerson, Lesem, Kaye, Hegg & Brewerton, 1989), obsessive-compulsive disorder (Kahn, van Praag, Wetzler, Asnis & Barr, 1988), chronic fatigue syndrome (Demitrack et al., 1992), panic disorder (Lesch et al., 1992), suicide (Arora & Meltzer, 1989), depression (Kahn, Wetzler, Asnis, Papolos & van Praag, 1990) and anxiety (Charney, Woods, Krystal & Heninger, 1990a, Eison & Eison, 1994) (for review see Lopez-Ibor, 1988).
The brain stem dorsal raphe and median raphe nuclei provide considerable innervation to numerous central sites including the cerebral cortex, the hypothalamus, the thalamus, the basal ganglia and various limbic areas (i.e., amygdala, hippocampus, and septum) (Azmitia & Segal, 1978; Molliver, 1987). Particularly high levels of 5-HT in limbic forebrain areas and the behavioural consequences of central 5-HT alterations (Chopin & Briley, 1987) have prompted speculation that 5-HT may be involved in emotional expression (Kahn et al., 1988). In this respect, anatomical, neurochemical, electrophysiological and behavioural data support the notion that 5-HT neurotransmission may be important in the regulation and/or manifestation of anxiety (Taylor, 1990). The identification of four serotonergic receptor subtypes (5-HT₁, 5-HT₂, 5-HT₃ and 5-HT₄) (Glennon, 1990) has led to various hypotheses regarding the role of 5-HT neurons in anxiety and the therapeutic actions of anxiolytic drugs at these respective 5-HT receptor sites.

Numerous clinical and preclinical studies employing 5-HT₁A agonists and partial agonists including buspirone, ipsapirone and gepirone have revealed that these agents are anxiolytic in humans (Lesch & Osterheider, 1991; Taylor, 1990; Traber & Glaser, 1987) and in infrahuman subjects (Briley, Chopin & Moret, 1991; Chopin & Briley, 1987; Critchley, Njung’e & Handley, 1992; Schreiber & De Vry, 1993). However, weak and inconsistent anxiolytic effects are also reported in the evaluation of 5-HT₁A receptor agonists (Handley, McBlane, Critchley & Njung’e, 1993). In addition, the behavioural results of animal studies in which benzodiazepines elicit anxiolytic effects do not correlate well with investigations employing selective 5-HT₁A agonists (Sanger, Perrault, Morel,
Joly & Zivkovic, 1991) The presence of pre- and post-synaptic 5-HT_{1A} receptors in terminal regions of the raphe system may account for the equivocal nature of some of the data derived from animal experimentation (Oakley & Tyers, 1992). Moreover, it should be considered that an animal model may assess diverse aspects of anxiety which may be mediated by specific 5-HT receptor subtypes and/or different neurotransmitter systems (Barrett, 1991; Handley & McBlane, 1993).

While the 5-HT_{1A} receptor has been identified in all primates, the 5-HT_{1B} receptor exists only in rodents and the 5-HT_{1D} receptor is found in non-rodent species (e.g., humans) (Hoyer & Middlemiss, 1989). The rat and mouse 5-HT_{1B} receptors are regarded as species equivalents of the 5-HT_{1D} receptor in man (Clarke, 1992; Humphrey, Hartig & Hoyer, 1993). Recently, Benjamin, Lal and Meyerson (1990) reported that systemic administration of the 5-HT_{1B} selective agent, trifluoromethylphenylpiperazine (TFMPP), produced anxiogenic effects in the elevated plus maze in mice. Nevertheless, experimental evidence regarding the involvement of 5-HT_{1B} or _1D_ receptors in anxiety is limited. Such ambiguity may be due, in part, to a current lack of specific agonists and antagonists for either of these respective receptors (Broekkamp, Berendsen, Jenck & Van Delft, 1989; Oakley & Tyers, 1992).

Interestingly, administration of the 5-HT_{1C} agonist, m-chlorophenylpiperazine (mCPP), also a major metabolite of the antidepressant trazodone, provoked anxiogenic effects in panic patients and healthy volunteers (Charney, Woods, Goodman & Heninger, 1987). Likewise, intraperitoneal administration of mCPP in rodents enhanced anxious behaviour in the light-dark box (Griebel, Misslin, Pawlowski & Vogel, 1991) and in the
social interaction test (Kennett, Whitton, Shah & Curzon, 1989). These data should be considered cautiously, however, since mCPP has been reported to have considerable affinity for the 5-HT_{1B} and the 5-HT_{3} receptor as well (Curzon et al., 1991). Nonetheless, various 5-HT_{1C} receptor antagonists (e.g., mianserin, ICI 169 369, LY 53857) were found to produce anxiolytic effects in the rat social interaction model of anxiety (Kennett, 1992).

Administration of relatively selective 5-HT_{2} receptor antagonists, ritanserin, ketanserin and ICI 169369, produced anxiolytic effects in the elevated plus maze and light-dark box models of anxiety (Marsden, 1989). In other paradigms involving conflict procedures, however (e.g., potentiated startle), these antagonists were generally ineffective (Broekkamp et al., 1989; Oakley & Tyers, 1992). While few clinical trials have been undertaken with 5-HT_{2} antagonists, preliminary data indicate that ritanserin may have some anxiolytic efficacy in generalized anxiety (Ceulemans, Hoppenbrouwers, Gelders & Reyntjens, 1985; Deakin, Guimaraes, Wang, Hellewell & Hensman, 1991).

Studies investigating the role of the 5-HT_{3} receptor in anxiety have revealed inconsistent results (Rodgers, Cole & Tredwell, 1995). Anxiolytic responses to the 5-HT_{3} antagonists zacopride, ondansetron, granisetron and tropisetron are evident in tasks assessing unconditioned behaviour in animals (e.g., light-dark exploratory test, elevated plus maze) (Greenshaw, 1993; Young & Johnson, 1991a), but not in shock-conflict or punishment paradigms (Clarke, 1992; Nevins & Anthony, 1994). The basis for such experimental discrepancies is not immediately apparent, although Nevins and Anthony (1994) proposed that a lower shock intensity, than is traditionally used during fear conditioning, may be necessary to detect anxiolytic effects with 5-HT_{3} antagonists in
models employing shock. Based on studies evaluating the ability of ondansetron, zacopride, granisetron, diazepam and buspirone to reduce the fear-potentiated startle response in the rat, these investigators suggest that different conditioning protocols may produce qualitatively different anxiety states, which may be differentiated by 5-HT₁ antagonists (Nevins & Anthony, 1994, see also Meneses & Hong, 1993) Few clinical studies have been completed with 5-HT₁ antagonists to date, but in a recent report, the selective 5-HT₁ antagonist, tropisetron produced significant dose-dependent positive effects on anxiety related outcome measures in a group of outpatients with generalized anxiety (Lecrubier, Puech, Azcona, Bailey & Lataste, 1993).

It should be emphasized that 5-HT₁ receptor activation enhanced the release of CCK-like immunoreactivity from the cerebral cortex and nucleus accumbens in the rat (Paudice and Raiteri, 1993). In contrast, the 5-HT₁ antagonists, ondansetron and tropisetron, prevented the 5-HT₁-evoked release of CCK in the frontal cortex of rats implanted with microdialysis probes (Raiteri, Paudice & Vallebuona, 1993). Moreover, in another recent study employing guinea pigs, increased extracellular levels of 5-HT in the prefrontal cortex following exposure to the elevated plus maze were potentiated by the CCK₈ receptor agonist, BOC-CCK-4, and prevented by pretreatment with the selective CCK₈ antagonist, L365.260 (Rex, Fink & Marsden, 1994b). These data suggest an interaction between 5-HT and CCK in limbic and cortical areas which may contribute to the regulation or expression of anxiety.

Alterations in the 5-HT system will likewise induce changes in central NE activity (Kahn et al., 1988). Activation of serotonergic neurons projecting from the dorsal raphe
nucleus to the LC inhibits firing of the LC (Segal, 1979). Charney et al. (1990a) proposed that 5-HT associated decreases in LC neuronal activation may be mediated by the 5-HT₂ receptor, while Chiang and Aston-Jones (1993) suggested 5-HT₂ agonists influence LC indirectly, through tonic activation of inhibitory GABAergic input to the LC. Moreover, the anxiolytic effects of the α₂-NE agonist, clonidine, may not be a product of reduced NE activity alone, but also of an attenuation of 5-HT neuronal activity through stimulation of α₂-NE adrenoceptors situated on 5-HT neurons and/or terminals (Marsden, 1989). These interactions between 5-HT and NE and, consequently, CCK may play a prominent role in the expression of anxiety.

Central 5-HT activity may also be modulated by benzodiazepines. In fact, benzodiazepines, through GABAergic mechanisms, typically inhibit activity of dorsal raphe neurons (Gallager, 1978) and, as a result, decrease central 5-HT turnover (Taylor, 1990; Wise, Berger & Stein, 1972). Interestingly, exposure of rats to the elevated plus maze or to the social interaction test provoked a significant increase in extracellular 5-HT levels in the hippocampus (Wright, Upton & Marsden, 1992) and a decrease in cortical GABA function (File, Zangrossi & Andrews, 1993). In addition, Pie et al., (1989) reported that diazepam or flurazepam inhibited 5-HT release from the ventral hippocampus and this effect was reversed by the benzodiazepine antagonist Ro 15-1788 (Pie, Zetterstrom & Fillenz, 1989). Although the relevance of these effects for the anxiolytic profile of benzodiazepines is unclear, the possibility remains the benzodiazepines may be effective in the treatment of anxiety, in part, through an influence on 5-HT neurotransmission.
Taken together, a concise accounting of the role of 5-HT in anxiety is difficult, but typically, the action of putative 5-HT receptor mediated anxiolytics is to decrease 5-HT neuronal function, particularly in cortical and limbic areas of brain (Marsden, 1989). Such an explanation may be overly simplistic, however. It is likely that the expression or attenuation of anxiety follows from interactions between multiple neurochemical systems (Barrett & Vanover, 1993; Salzman et al, 1993). Accordingly, anxiety disorders in humans (e.g., generalized anxiety disorder, panic disorder, obsessive-compulsive disorder, posttraumatic stress disorder) likely arise from disparate neurochemical imbalances (Barrett, 1991; Broekkamp et al., 1989) and animal models of anxiety may assess diverse aspects of anxiety, to which 5-HT may contribute, in only some instances (Gyertyan, 1992; Handley & McBlane, 1993).

STRESSORS AND ANXIETY

Numerous investigations have revealed that aversive life events are often associated with the onset of anxiety disorders. For instance, Raskin et al (1982) reported that the stress of separation (e.g., divorce, leaving home) could initiate or exacerbate anxiety in individuals with panic or generalized anxiety disorder (Raskin, Peeke, Dickman & Pinsker, 1982), while other investigators have noted that a recent major loss (e.g., death) or a potentially threatening event (e.g., illness) may play a role in the precipitation of panic symptoms (Faravelli, 1985; Servant, Bailly, Allard & Parquet, 1991). In addition, life events may have considerable psychological and/or emotional impact on vulnerable individuals who view situational variables as uncontrollable. In effect, the perception of the uncontrollable nature of a stressor, rather than the impact of a stressor, may contribute
to the induction of anxiety (Roy-Byrne, Geraci & Uhde, 1986) For instance, Dalgard, Bjork and Tambs (1995) detected a significant relationship between negative life events, social support and mental health among individuals who perceived themselves unable to control situations in their lives. The combination of the feeling of powerlessness and lack of support in the face of a stressor identified a high-risk group with respect to mental health. On the other hand, subjects who believed they were able to maintain some control over life events, required significantly less social support when confronting similar stressors.

In addition to the observation that stressful life events often precede anxiety in humans, an association between stressors and anxiety has also been demonstrated in numerous animal models of anxiety (Carli, Prontera & Samanin, 1989; McBlane & Handley, 1994). The avoidance of threatening and potentially threatening stimuli serves an adaptive function in infrahuman species. Animal models of anxiety typically rely on such behaviour in paradigms employing punishment, conditioned fear or novelty (Thiebot, 1993). Moreover, many models are based on a conflict between motivations which generates an approach-avoidance situation (Robins, 1992). For example, aversive stimuli including electric shock, open spaces, elevated heights or unfamiliar environments may be paired with food, water or a non-threatening, familiar environment (Trullas & Skolnick, 1993). The Geller-Seifter conflict procedure and the Vogel punished drinking paradigm assess an animal’s propensity to emit a response which is rewarded with food or water when the response is simultaneously punished with footshock (Thiebot, 1993). Likewise, the conditioned emotional response test and the potentiated startle paradigm are based on
classical conditioning in which the pairing of a neutral stimulus (e.g., tone or light) with an aversive one (e.g., shock) leads to the disruption of reinforced responding, or an enhanced auditory startle response upon the presentation of the neutral stimulus (File, 1985). Anxiolytics ordinarily reinstate suppressed responding for reinforcement (Lal & Emmett-Oglesby, 1983) and reduce fear potentiated startle (Davis, 1992).

One of the major criticisms of models based on punishment or conditioned fear is the possible confounding influence of the application of noxious stimuli or the deprivation of food or water (Gyertyan, 1992; Trullas & Skolnick, 1993). Examples of more ethologically relevant versions of conflict models include the social interaction test, the elevated plus maze and the light-dark box paradigm (File, 1985; Lister, 1991). The social interaction test developed by File and Hyde (1978) employs novelty and increased illumination as anxiogenic stimuli and assesses, among other behaviours, the social interplay between rats in various testing conditions (File, 1992). The elevated plus maze, a plus-shaped apparatus with two opposing open and two opposing enclosed arms, exploits the conflict between an animal's innate tendency to explore and an aversion to open spaces and elevated platforms. Anxiolytics generally increase the time animals spend in the open arms of the maze (Pellow & File, 1986). Finally, the two compartment test or light-dark box paradigm (Crawley & Goodwin, 1980), makes use of the conflict between an organism's propensity to explore and the innate aversion to bright, open spaces. The apparatus consists of a large, brightly lit area and a smaller, dark compartment with an opening between the two. Behaviours typically assessed in this model include transition frequency and amount of time spent in the dark and light portions of the box. Animals
which typically spend most of their time in the smaller, dark area of the test arena spend
significantly more time exploring the larger, bright compartment following the
administration of anxiolytics and less time in the light area following anxiogenics
The foregoing survey of animal models of anxiety is far from exhaustive. Further examples
of recently proposed models include the rat pup vocalization model (Insel & Winslow,
1991), separation induced hypothermia (Borsini, Lecci, Volterra & Meli, 1989),
thigmotaxis (Treit & Fundytus, 1989), the open field drink test (Stout & Weiss, 1994), the
anxiety/defense test battery (Blanchard et al., 1993b) and conditioned defensive burying
(Treit, 1985).

It is of considerable interest that many investigators have suggested some animal
models of depression may also be suitable for the investigation of anxiety (Hommer,
Skolnick & Paul, 1987; Thiebot, 1993; Van Dijken, Mos, Van der Heyden & Tilders,
1992). For example, exposure to inescapable footshock produces deficits in learning to
avoid or escape shock in a shuttle box (Anisman, de Catanzaro & Remington, 1978; Maier
& Seligman, 1976), results in immobility in the ‘behavioural despair’ or forced swim test
(Prince & Anisman, 1984) and produces anhedonia, as evidenced by reduced responding
for ICSS (Zacharko & Anisman, 1991). It is suggested that these behavioural alterations
may model some symptoms of human depression (Anisman & Zacharko, 1990; Weiss,
1991). Moreover, it has been postulated that emotional reactions, including anxiety or fear
may be involved in the development of these behavioural disturbances (Drugan, Ryan,
Minor & Maier, 1984; Prince, Collins & Anisman, 1986; Van Dijken, Tilders, Olivier &
The induction of fear or anxiety upon exposure to uncontrollable footshock is supported by the observation that benzodiazepine administration prior to stressor application prevented the appearance of shuttle escape deficits (Drugar et al., 1984; Thiebot, 1993) and disrupted the initial motor invigoration in the forced swim task normally engendered by shock treatment (Prince et al., 1986). Moreover, the benzodiazepine inverse agonist, FG 7142, like uncontrollable shock, induced escape deficits in the shuttle task and this effect was blocked by acute benzodiazepine pretreatment and the benzodiazepine antagonist, Ro 15-1788 (Hommer et al., 1987). Likewise, administration of FG 7142 produced decreases in social interaction similar to those detected following inescapable shock presentation and these decreases in social interaction were blocked by the administration of the benzodiazepine antagonist, flumazenil (Short & Maier, 1993). Interestingly, these results have led Short and Maier (1993) to suggest that an endogenous benzodiazepine receptor inverse agonist may be released during inescapable shock which is involved in producing subsequent behavioural alterations.

In addition to producing behavioural deficits, uncontrollable stressors also influence central neurochemical activity. For example, there are considerable data linking uncontrollable stressors to alterations in the levels of central NE (Weiss & Simson, 1984; Weiss, 1991), DA (Deutch & Roth, 1991; Deutch, Tam & Roth, 1985), and 5-HT (Dunn, 1988b; Kawahara, Yoshida, Yokoo, Nishi & Tanaka, 1993), as well as to the modulation of the benzodiazepine-GABA receptor complex (Drugar & Holmes, 1991). As there are extensive reviews assessing stressor induced NE, DA and 5-HT alterations (see Weiss,
1991, Roth, Tam, Ida, Yang & Deutch, 1988, Chaouloff, 1993 respectively), only a brief synopsis will follow.

Experimental evidence suggests that central benzodiazepine (Rago, Kiivet, Harro & Pold, 1989) and GABA receptors (Kellogg, Inglefield, Taylor & Pleger, 1993) in the cerebral cortex and/or hippocampus are affected by stressor exposure, but there are discrepancies regarding the nature of such receptor alterations. For example, some laboratories have reported an increase in the density of benzodiazepine cortical receptors in the rat following forced swim stress (Rago et al., 1989, Motohashi, Okamoto, Osada & Yamawaki, 1993), while others have cited stressor induced decreases in the number of binding sites in the cerebral cortex and hippocampus (Medina, Novas, Wolfman, Levi de Stein & de Robertis, 1983). Similar inconsistencies are found in the literature with respect to changes in the GABA<sub>A</sub> receptor in response to stress (Drugan & Holmes, 1991). Factors which may account for these contrasting reports include differences in the properties of the stressors employed (e.g., type, chronicity, severity), the brain area under consideration, the species of animal tested, and the multiple sites at which GABA may exert its actions, among others.

In contrast to the discordance encountered with respect to stressor induced benzodiazepine-GABA alterations, there is a general consensus that exposure to acute, uncontrollable stressors leads to decreased levels of NE and increased concentrations of the metabolite, MHPG, in several CNS sites (Anisman & Zacharko, 1990; Weiss, 1991). Moreover, stressor severity affects the magnitude and duration of such amine alterations and may also influence the brain region in which enhanced NE turnover is apparent. For
example, stressors of moderate severity typically provoke marked changes in NE concentrations within the hypothalamus, amygdala and locus coeruleus (Iimori et al., 1982; Tsuda & Tanaka, 1985, Weiss, 1991). More severe stressors, however, may also affect NE levels in the frontal cortex and hippocampus (Tanaka et al., 1982).

In addition to provoking changes in NE, stressors also influence central DA activity and these alterations appear to be region specific. Typically, uncontrollable stressors readily provoke DA turnover in mesocorticolimbic structures including the prefrontal cortex, nucleus accumbens and VTA, but only limited DA changes are observed in nigrostriatal regions (Abercrombie, Keefe, DiFrischia & Zigmond, 1989; Zacharko & Anisman, 1991; Deutch, et al., 1985; Thierry, Tassin, Blanc & Glowinski, 1976). With more intense stressors however, increases in DOPAC have also been observed in the amygdala and striatum (Dunn, 1988a). Parenthetically, it should be noted that not all stressors are equally effective in increasing DA utilization and that some stressors have been reported to have no effect on DA turnover (e.g., noise stress) (Antelman et al., 1988).

In general, it would appear that more severe stressors are necessary to elicit 5-HT alterations than those which induce NE and DA changes (Dunn, 1988b, Inoue, Koyama & Yamashita, 1993). Acute footshock or restraint stress increases levels of 5-hydroxyindoleacetic acid (5-HIAA) in a number of brain regions including the prefrontal cortex (Shanks, Zalcman, Zacharko & Anisman, 1991), nucleus accumbens, amygdala (Inoue et al., 1993) and hypothalamus (Dunn, 1988b, Mitchell & Thomas, 1988). In addition, while many stress manipulations have produced little, or no alteration in levels of
5-HT itself, other investigators have recently reported increases in extracellular 5-HT levels in the amygdala and prefrontal cortex following psychological stress (Kawahara et al., 1993) and in the hypothalamus following immobilization stress (Shimizu, Take, Hori & Oomura, 1992).

There are numerous reports to support the contention that CCK may also participate in the behavioural and neurochemical response to stressors (Benoliel, Nevo, Becker, Lanfumey & Hamon, 1995, Harro, Vasar & Bradwejn, 1993; Seigel, Duker, Pahnke & Wuttke, 1987) For example, following exposure of animals to various durations of footshock (i.e., 2, 4, 10, 30 or 60 minutes), elevated CCK levels were detected in the posterior arcuate nucleus and the ventromedial, dorsomedial and anterior lateral hypothalamic areas (Seigel et al., 1987), as well as extrahypothalamic limbic sites (e.g., hippocampus, septum) (Siegel, Duker, Fuchs, Pahnke & Wuttke, 1985) Likewise, 30 minutes of restraint provoked significant release of CCK in the rat frontal cortex, which was prevented by prophylactic diazepam pretreatment (Benoliel et al., 1995) and brief restraint or an intraperitoneal injection led to significant increases in CCK-like immunoreactivity in the periaqueductal grey (Rosen, Brodin, Eneroth & Brodin, 1992). In addition, it has been suggested that the anticipation of stressful encounters may effect CCK release in humans. For instance, plasma CCK levels were elevated in marathon runners who were about to participate in a competitive run (Phillipp, Wilckens, Friess, Platte & Pirke, 1992). Taken together, these data suggest the activation of CCK systems, in addition to NE, DA, and 5-HT in response to stressor exposure.
Typically, stressor induced neurochemical alterations are transient (Anisman, et al, 1991). Nonetheless, exposure to an aversive stimulus may also have long term consequences. Indeed, the neurochemical and behavioural response to a stressor or pharmacological challenge may depend upon prior drug (e.g., amphetamine, cocaine) or stressor history, as well as the interval between initial challenge and re-exposure (Antelman, 1988, Antelman & Caggiula, 1990, Post, 1980). The application of an acute, brief, or intermittent stressor, for example, (e.g., restraint, footshock, injection procedures) has been reported to produce increased behavioural or neurochemical reactivity to the same or different stressor at a later date (sensitization) (Antelman, 1988). In addition, it has been demonstrated that behavioural and neurochemical correlates of sensitization are not only enduring, but may also increase with time following exposure to the initial sensitizing stimulus (Antelman & Caggiula, 1990, Robinson, 1988). Indeed, Paulson, Camp and Robinson (1991) report that behavioural sensitization to a challenge dose of amphetamine was not fully expressed until two weeks following the discontinuation of a chronic amphetamine schedule, but the enhanced stereotypic behaviours persisted for at least one year.

Although neurochemical sensitization is not unique to any particular transmitter system, considerable research has focused on alterations of DA neurotransmission in the induction of sensitization (Kalivas & Stewart, 1991). It was demonstrated, for instance, that footshock sensitized the cortical DA response to the same stressor ten days later (Caggiula, Antelman, Aul, Knopf & Edwards, 1989) and that presentation of cues previously paired with footshock enhanced DA release in the frontal cortex (Herman et al,
1982) and in the VTA (Deutch et al., 1985). In addition, both psychostimulants and stressors may provoke a behavioural and neurochemical sensitization through an enduring enhancement of central DA reactivity (Robinson, Angus & Becker, 1985; Steketee, Sorg & Kalivas, 1991). Indeed, it will be recalled that stressor exposure provoked increased dopamine release in the striatum, nucleus accumbens and frontal cortex (Abercrombie et al., 1989), and psychostimulant administration similarly increased extracellular dopamine concentrations in the nucleus accumbens and prefrontal cortex as measured by in vivo microdialysis (Sorg, 1992). Taken together, the data suggest that treatments which lead to behavioural sensitization may also lead to sensitization within the mesolimbic DA system as measured by augmented DA release (Steketee et al., 1992).

In addition to the detection of sensitization of DA activity, there is also considerable evidence favouring stressor induced sensitization of mechanisms subserving NE activity. For example, in animals previously exposed to an acute stressor, reexposure to a limited amount of the stressor increased NE utilization in the hypothalamus and hippocampus (Anisman & Sklar, 1979; Irwin, Ahluwalia, Zacharko & Anisman, 1986) and reexposure to cues associated with a stressor also increased NE utilization (Cassens, Roffman, Kuruc, Ursulak & Shildkraut, 1980). Moreover, acute tail shock produced a greater elevation in extracellular NE in the hippocampus of rats that had been previously exposed to cold stress than in controls which had not been stressed previously (Nisenbaum, Zigmond, Sved & Abercrombie, 1991). Interestingly, Camp et al. (1995) employed in vivo microdialysis to characterize NE neurotransmission after amphetamine pretreatment and challenge. Following 30 days of amphetamine withdrawal, basal
hypothalamic extracellular NE did not differ from controls, but amphetamine challenge provoked a significantly greater enhancement in hypothalamic NE release in rats which had undergone withdrawal (Camp, DeJonghe & Robinson, 1995) These data are consistent with the suggestion that alterations in NE neurotransmission may also contribute to behavioural and neurochemical sensitization.

It may be relevant to note that bidirectional cross-sensitization can be demonstrated between stressors and pharmacological agents (Hamamura & Fibiger, 1993; Robinson, 1988). In fact, sensitization to stressors and cross-sensitization between various stressors and drugs may have implications for some of the symptoms associated with anxiety and panic disorder (Antelman, 1988, Zacharko, Koszycki, Mendela & Bradwejn, 1995). In effect, initial encounters with aversive life events which result in the sensitization of a neurochemical mechanism may have a substantial influence on an organism’s vulnerability to subsequent behavioural disturbances (Zacharko & Anisman, 1989). Moreover, it will be recalled that CCK levels are altered by stressor exposure and that CCK may produce some of its anxiogenic effects through interactions with central GABA, NE, DA and 5-HT mechanisms. It should therefore be considered that CCK may modify the immediate and long term consequences of stressors through sensitization processes.

Indeed, in view of the fact that CCK administration and stressors may result in similar behavioural and/or neurochemical profiles, it is possible that cross-sensitization between stressor and CCK exposure may result in exacerbated anxiogenic reactivity.

The following series of experiments will evaluate: a) the immediate and long term behavioural consequences of different durations of uncontrollable footshock and restraint
stress in the light-dark box model of anxiety employing CD-1 mice, b) the influence of surgical stress on baseline levels of anxiety and on anxiogenic behaviour induced by exposure to uncontrollable footshock, c) the behavioural effects of intraventricular administration of varying doses of CCK-8S in the light-dark box, d) whether pharmacological sensitization can be detected upon a subsequent challenge with CCK-8S and d) whether behavioural cross-sensitization between stressors (footshock or restraint) and CCK-8S exposure might result in subsequent exacerbated anxiogenic reactivity in CD-1 mice tested in the light-dark box.
EXPERIMENTS 1A & 1B

It will be recalled that exposure to uncontrollable aversive events promotes behavioural disturbances in numerous paradigms and that the nature and duration of a stressor can influence the magnitude and/or time course of behavioural pathology (Anisman & Zacharko, 1990; Weiss & Simpson, 1984). It should be considered that encounters with uncontrollable life events have also been associated with the induction of fear and/or anxiety (Drugar et al., 1984) and Prince et al., (1986) have suggested that such anxiogenic effects may be reflected in behavioural alterations. Consistent with this position, McBlane & Handley, (1994) reported that one hour of restraint stress produced a marked anxiogenic effect in rats tested in the elevated plus maze, both immediately and 24 hours following release from restraint. Comparable results were reported by Rodgers & Cole (1993a) in mice immediately following defeat by an aggressive conspecific.

Experiments 1a and 1b were designed to evaluate the anxiogenic effects of uncontrollable footshock and restraint stress in the light-dark box paradigm. The parameters of stressor imposition were varied in each of the Experiments and the immediate and long term behavioural consequences of stressor imposition were evaluated in CD-1 mice.

Method

Subjects

Forty-eight naive, male, CD-1 mice were obtained from Charles River Canada (St. Constant, Quebec) at five weeks of age. Animals were acclimatized to the animal facility until they were the appropriate age for testing (approximately 3 months). All mice were
housed four per cage and maintained on a 12 hour light-dark cycle (light on 7:00 am until 7:00 pm) Food and water were available ad libitum.

**Apparatus**

The light-dark test apparatus consisted of a rectangular Plexiglas box, 20 cm x 47 cm x 20 cm high, with the light section, painted with non-toxic white enamel, occupying two-thirds of the box and the dark section, painted with non-toxic black enamel, comprising the remaining third. The two chambers were separated with a Plexiglas partition (painted the appropriate colour on each side) with an opening 12.5 cm x 5 cm high allowing non obstructed passage from one section of the apparatus to the other. A 60 watt light bulb, placed 10 cm above the center of the white section provided illumination in that chamber, while the top of the dark section was covered with red, translucent Plexiglas.

The shock apparatus (9.5 cm x 28 cm x 16 cm high) consisted of black Plexiglas walls and a floor of stainless steel rods, connected in series, spaced 1 cm apart. Footshock (150 uA, 60 x 6 sec duration, with 59 sec. inter-trial interval) was delivered by a microcomputer controlled 3000 V source (Science Technology Center, Carleton University). Identical boxes were used in the no shock condition.

The restraint apparatus was a clear semicircular Plexiglas tube, measuring 2.5 cm wide, with slots at regular intervals for the insertion of a small rectangular door which enclosed the animal in the tube from behind. Appropriate breathing holes were located at the front of the apparatus. Once the mouse was positioned in the restraint tube, its tail was taped to further restrict movement within the tube.
Procedure

All subjects in Experiments 1a and 1b were tested in the light-dark box for 10 minutes over three consecutive days between 9:00 a.m. and 12:00 p.m. Each mouse was removed from its home cage and placed in the center of the light chamber facing the opening in the partition to the dark section of the box. The latency for the mouse to enter the dark chamber, the number of transitions between chambers and the time spent in the dark and light chambers were recorded during each session. An animal was considered to have entered a chamber and timing commenced when all four paws were positioned in the chamber.

Following a 3 day Baseline anxiety assessment, mice in Experiment 1a were assigned to one of four stressor conditions: No footshock, 15 footshocks, 30 footshocks or 60 footshocks (n=6 per group). On the Immediate test day, mice were exposed to the respective stressor conditions or were maintained in an identical apparatus, but the stressor was withheld. Immediately following the stressor session, mice were evaluated in the light-dark box employing the latency, transition and cumulative scores previously outlined. A final test in the light-dark box was conducted one week following initial stressor exposure.

In Experiment 1b, following the three day Baseline anxiety assessment, animals were assigned to one of four treatment conditions. No restraint, 15 minutes of restraint, 30 minutes of restraint or 60 minutes of restraint (n=6 per group). Mice were tested in the light-dark box immediately and one week following initial stressor application. Following completion of Experiments 1a and 1b, animals were sacrificed in a CO2 chamber.
Results

Experiment 1a

Anxiety Index: An ‘Anxiety Index’ (AI), employing both the total time in the light compartment of the apparatus and the number of transitions between compartments was calculated for each subject’s test session in each of Experiments 1a and 1b. The AI was defined as the total time, in seconds, spent in the light section of the box divided by the number of transitions into the light compartment (i.e., average time in light per entry). Decrements in AI, as indicated by reduced time in light per entry, were associated with augmented anxiety levels, while increments in AI corresponded to a reduction in anxiety.

Baseline Data: Baseline AI scores were analyzed over 3 consecutive test days to ensure the four treatment groups did not differ on anxiety scores prior to stressor exposure in the light-dark apparatus. The analysis of variance of the AI scores before stressor exposure revealed the No shock, 15 shock, 30 shock and 60 shock groups did not differ significantly in AI levels before shock treatment ($F_{(6,40)} = 0.44$, $p > 0.05$ for the Day x Shock interaction). In addition, the analysis of variance of baseline AI scores failed to reveal a significant main effect for Day ($F_{(2,40)} = 1.13$, $p > 0.05$).

Insert Figure 1a about here

Post Treatment Data: An analysis of variance of the AI scores from the immediate and the one week post stressor tests among mice exposed to No shock, 15 shocks, 30 shocks or 60 shocks revealed a significant Day x Shock interaction ($F_{(3,20)} = 11.57$, $p < 0.01$).
Subsequent simple effects analysis and Newman Keuls multiple comparisons indicated a significant difference on AI scores between the four shock groups on the immediate test day ($F_{3,20} = 28.21$, $p < 0.01$), such that mice exposed to 15, 30 or 60 uncontrollable footshocks differed significantly on AI from mice exposed to No Shock, but the three shock groups did not differ from one another. Such behavioural effects were restricted to the immediate post stressor interval as significant differences were not detected on AI scores one week following initial stressor exposure.

Insert Figure 1b about here

Experiment 1b

**Baseline Data.** The Baseline AI scores of mice were analyzed as described in Experiment 1a. The analysis of variance of AI scores over the three days prior to stressor exposure revealed the No restraint, 15, 30 and 60 minute restraint groups did not differ significantly on AI levels prior to immobilization stress ($F_{6,40} = 0.43$, $p > 0.05$ for the Day x Restraint interaction). In addition, the main effect of day ($F_{2,40} = 1.49$, $p > 0.05$) failed to reach an appropriate level of statistical significance, suggesting that anxiety scores were not influenced by repeated testing in the light-dark box.

Insert Figure 2a about here
Figure 1a  (Upper graph) Mean (± S E M ) Baseline anxiety index (Time(sec.)/Transition) of CD-1 mice tested for 10 minutes in the light-dark apparatus on 3 consecutive days prior to exposure to 0, 15, 30 or 60 uncontrollable footshocks (n=24).

Figure 1b  (Lower graph) Mean (± S E M ) anxiety index (Time(sec.)/Transition) for CD-1 mice exposed to an acute session of 0, 15, 30 or 60 uncontrollable footshocks and assessed in the light-dark box (10 minute session) immediately and one week following stressor exposure (n=6/group).
Post Treatment Data  An analysis of variance of the AI scores on the immediate and one week post stressor tests among mice exposed to No restraint, 15, 30 or 60 minutes of restraint stress revealed a significant Day x Restraint interaction ($F_{(3,20)} = 3.81, p < 0.05$). Subsequent simple effects analysis and Newman Keuls multiple comparisons detected a significant difference on AI scores between the four treatment groups on the Immediate test day ($F_{(3,20)} = 3.18, p < 0.05$) such that animals in the 30 minute restraint group differed significantly from those exposed to No stress. The 15 and 60 minute restraint groups did not differ significantly from either the No stress or the 30 minute restraint group. There were no significant differences on AI scores among the No stress, 15, 30 or 60 minute restraint groups one week following initial immobilization stress.

Insert Figure 2b about here

Discussion

The experimental data pertaining to the use of transition scores and time in light as a reflection of anxiety in the light-dark paradigm have produced variable results. For example, some investigators have reported increases in transition frequency and time in light following anxiolytic administration (Belzung et al., 1987; de Angeles, 1992; Shimada et al. 1995); other laboratories have reported alterations in the former, but not the latter (e.g., Crawley, 1981; Crawley & Goodwin, 1980), while still others have demonstrated the converse following anxiolytic administration (Belzung, 1992; Costall, Jones, Kelly, Naylor & Tomkins, 1989; Kilfoil et al., 1989; Onaivi & Martin, 1989; Young & Johnson,
**Figure 2a:** (Upper graph) Mean (± S.E.M) Baseline anxiety index (Time/sec /Transition) among CD-1 mice tested for 10 minutes in the light-dark apparatus on 3 consecutive days prior to exposure to 0, 15, 30 or 60 minutes of restraint (n=24).

**Figure 2b:** (Lower graph) Mean (± S.E.M) anxiety index (Time/sec /Transition) among CD-1 mice exposed to an acute session of 0, 15, 30 or 60 minutes of restraint stress and assessed in the light-dark box (10 minute session) immediately and one week following stressor exposure (n=6/group).
Accordingly, it was considered that the behavioural expression of anxiety may be more adequately assessed by employing a ratio of cumulative time in light and transition frequency. The ratio of these two measures, the anxiety index (AI), was operationalized as the mean time in the light compartment per entry during a test session. In fact, employment of ratio measures to depict spatial and temporal distributions of behaviour in anxiety paradigms is not unfounded, although use of such an index has been limited (see Rodgers & Cole, 1993a, b).

In addition to variations associated with the choice of specific behavioural measures in assessing anxiety, the effects of both repeated testing in the light-dark box on behavioural change, as well as pharmacological challenge, have also been variable. While some laboratories have tested animals in the light-dark paradigm on three (Blumstein & Crawley, 1983) or four consecutive days (Onaivi & Martin, 1989), others have argued that repeated apparatus exposure influences subsequent expression of anxiogenic behaviour and exposure of animals to the apparatus should be limited to a single session (Barry, Costall, Kelly, Naylor & Onaivi, 1987, Rodgers & Shepherd, 1993). It should be considered that such methodological variance profoundly influences the behavioural response profiles observed in paradigms which assess anxiety. Moreover, the considerable decrease in either time or transition frequency with repeated testing largely preclude the imposition of additional experimental manipulation, due to the induction of floor effects and may confound an interpretation of anxiety, as the decreases may also reflect habituation to the novelty of the test environment (Barry et al., 1987, Blumstein &
Crawley, 1983). It is notable that, in the present investigation, the adoption of the AI prevented any significant variations in anxiety with repeated testing.

It will be recalled that exposure of animals to uncontrollable aversive events produces behavioural deficits in a number of animal paradigms including shuttle-escape (Anisman & Sklar, 1979; Drugan et al., 1984), forced swim (Prince et al., 1986), intracranial self-stimulation (Zacharko, et al., 1983), the elevated plus maze (McBlane & Handley, 1994), open field exploration (Carli et al., 1989), social interaction (Short & Maier, 1993), and the light-dark paradigm (Cancela, Breganzio & Molina, 1995; Guanowsky & Seymour, 1993), among others. It has been suggested that stressor provoked behavioural alterations in these paradigms may be associated with the induction of anxiety (Drugan et al., 1984; Van Dijken et al., 1992a, b). For example, 60 inescapable footshocks (Steenbergen, Heinsbroek, Van Hest & Van de Poll, 1990) and one hour (but not 15 minutes) of restraint (McBlane & Handley, 1994) were reported to provoke anxiogenic behaviour in rats in the elevated plus maze. Likewise, one hour, but not 15 or 30 minutes of restraint stress decreased the number of transitions between compartments in the light-dark box (Guanowsky & Seymour, 1993) Consistent with such observations, mice in Experiment 1a, exposed to an acute session of uncontrollable footshock, exhibited significant increases in anxiety, as revealed by decreased AI scores in the light-dark box, following 15, 30 or 60 footshocks. In addition, 15, 30, or 60 uncontrollable footshocks provoked anxiogenic profiles that were behaviourally indistinguishable from one another. In contrast to the above reports however, 30 minutes, but not 15 or 60 minutes of
immobilization produced maximal anxiogenic responses in CD-1 mice tested in the light dark box (Experiment 1b).

Numerous investigations have demonstrated that the behavioural alterations following exposure to uncontrollable footshock are associated with perturbations of central neurochemical activity (Anisman & Zacharko, 1990). Indeed, changes in DA (Deutch et al., 1985), NE (Weiss, 1991), 5-HT (Shanks, et al., 1991; Plaznik, Tamborska, Hauptmann, Bidzinski & Kostowski, 1988), GABA (Biggio et al., 1990; Drugan & Holmes, 1991) and CCK (Siegel et al., 1987), among others, may underlie the expression of anxiety. It is notable that region specific alterations in amine levels often occur rapidly upon exposure to footshock and are maintained during the stressor session (e.g., DA, Fadda et al., 1978; and CCK, Seigel et al., 1987). It might be considered, therefore that the neurochemical alterations attending stressor presentation, may correlate with the induction of anxiogenic behaviour demonstrated following 15, 30 or 60 uncontrollable footshocks.

While exposure of animals to the respective footshock parameters did not differentiate animals behaviourally in the light dark box paradigm, immobilization stress produced an anxiogenic response profile in the light-dark box which varied directly with the duration of the restraint session. Maximal behavioural changes in the AI were observed immediately following 30 minutes of restraint, while anxiety levels of mice immobilized for 15 or 60 minutes did not differ from non-stressed animals. The post restraint AI profile of CD-1 mice parallels the profile of self stimulation performance from the nucleus accumbens among BALB/cByJ mice exposed to immobilization stress.
Specifically, 30 minutes of restraint induced more pronounced behavioural changes relative to the more protracted stressor session of 60 minutes (Dickson, 1990). Taken together, these data suggest that paradigms assessing either motivational alteration or anxiogenic variations are sensitive to the differential influence of varying durations of immobilization stress.

The anxiety profiles evident among CD-1 mice immediately following various durations of restraint stress are particularly interesting in view of the within session neurochemical adaptation apparent during restraint (Puglisi-Allegra, Imperato, Angelucci & Cabib, 1991; Roth et al, 1988; Tanaka et al., 1982). For example, immobilization increased DOPAC concentrations in both the prefrontal cortex and nucleus accumbens in rats which were dependent upon the duration of the restraint session (Roth et al, 1988). Dopamine metabolite elevations were apparent in the frontal cortex within 5 minutes of immobilization stress, peaked at 30 minutes, and then decreased with continued restraint. In the nucleus accumbens, in contrast, increases in DOPAC concentration appeared within 30 minutes and were maintained throughout the restraint session. Similarly, Puglisi-Allegra et al. (1991) reported a considerable increase in the release of DA in the prefrontal cortex and nucleus accumbens in rats 20 to 40 minutes after the onset of restraint. However, DA levels in both regions returned to basal levels within 60 minutes of stressor onset (Imperato, Puglisi-Allegra, Casolini & Angelucci, 1991; Puglisi-Allegra et al., 1991). Accordingly, the behavioural expression of significant anxiety following 30 minutes, in contrast to 15 or 60 minutes of restraint in the light dark paradigm, appears to parallel the induction of appreciable elevations of DOPAC, while the decline of DOPAC
concentrations to basal levels may underlie the alleviation of anxiety exhibited following 60 minutes of restraint.

It should be emphasized that alterations in neurotransmitter turnover which appear to be dependent upon immobilization duration are not restricted to the DA system. In fact, immobilization stress evoked marked increases in the release of NE in the paraventricular nucleus of the rat hypothalamus which were maximal 30 minutes after restraint onset (Pacak, Palkovits, Kvetnansky, Kopin & Goldstein, 1993), while in the lateral septum and stria terminalis, NE levels peaked 20 minutes following immobilization onset (Saavedra, 1982). Likewise, duration dependent variations in NE turnover induced by restraint stress were also detected in the hypothalamus, amygdala, thalamus, hippocampus, and cerebral cortex of rats, with the most rapid and marked NE response observed in the hypothalamus and amygdala (e.g., decreased NE and increased MHPG levels within 30 minutes of immobilization), and a delayed NE turnover in the remaining regions (i.e., 60 to 120 minutes post stressor onset) (Tanaka et al., 1982).

It is notable that various regional neurochemical changes provoked during restraint may be paralleled by the behaviour of the animal. For example, during the early phases of restraint, animals typically exhibit vigorous struggling, vocalization and defecation. Such agitated behaviour dissipates gradually to a period of relative quiescence (Glavin, 1985). Interestingly, Keim and Sigg (1978) reported that struggling typically subsided after 30 minutes of restraint (see Fig. 2, Keim & Sigg, 1978). It might be considered that this decrease in behavioural agitation corresponds to the neurochemical adaptation reported to commence after approximately 30 minutes of restraint (Puglisi-Allegra et al., 1991, Pacak,
et al., 1993) and the observed decrease in anxiety in the light dark box observed following 60 minutes of immobilization.

It was not entirely unexpected that uncontrollable footshock and immobilization produced different anxiety profiles in the light dark paradigm, in view of the observation that the stimulus properties associated with these stressors are fundamentally different from one another. For example, footshock consists of an intermittent presentation of a relatively noxious stimulus which promotes motor excitation (i.e., running and jumping), while restraint is a continuous stimulus which restricts movement (Puglisi-Allegra, 1991). It has been suggested, however, that the two stressors may share a common element, anxiety, which may contribute to the initiation of similar behavioural and neurochemical alterations (Roth et al., 1988).

It will be recalled that stressor provoked behavioural deficits are often no longer evident one week following initial stressor application (e.g., self-stimulation from the nucleus accumbens (Dickson, 1990; Zacharko, Lalonde, Kasian & Anisman, 1987). Likewise, AI scores among CD-1 mice assessed in the light dark box returned to baseline levels one week following exposure to either uncontrollable footshock or immobilization stress. It should be noted however, that relatively protracted behavioural changes have been reported with self-stimulation from the ventral tegmental area (Maddeaux & Zacharko, 1992) and in open field activity (VanDijken et al., 1992a) following stressor exposure. While AI scores in the present investigation returned to pre-stressor levels, the variations in anxiety evident in the immediate post stressor interval indicate the nature and duration of the stressor may have influenced the composition and timecourse of
neurotransmitter release. Therefore, although the release of DA and NE, among other neurotransmitters, may subserve the observed behavioural alterations in anxiety, the underlying patterns of release among animals exposed to uncontrollable footshock may have been fundamentally different from those of animals exposed to restraint stress. Accordingly, mice exposed to diverse stressors or to various durations of the same stressor may demonstrate divergent behavioural profiles upon subsequent exposure to stressor or pharmacological challenge. This proposition will be examined in Experiment 4.

Taken together, it appears the nature and the duration of an aversive event may influence subsequent anxiogenic behaviour, the composite and timecourse of central neurotransmitter alterations and may also have a predisposing influence on subsequent behavioural and neurochemical responses to aversive events.
EXPERIMENT 2

The expression of behavioural pathology following exposure to uncontrollable stressors appears to be influenced, not only by the nature and duration of the stressor, but also by the psychosocial setting upon which the stressor is applied, as well as the previous stressor history of the organism (Anisman & Sklar, 1981). In this respect, there is some evidence to suggest that the stress associated with isolation housing may have profound behavioural and neurochemical consequences (Brain, 1975; Brain & Benton, 1979). In particular, isolated animals exhibit significantly greater increases in locomotor activity compared to group housed rats in a novel environment (McCloskey, Ketteler, Fadayel, Schmidt & Kehne, 1994). Moreover, rats isolated for 7 days (Vasar et al., 1993), or for as little as two hours (Maisonnette, Morato & Brandao, 1993) displayed increased anxiogenic behaviour in the elevated plus maze relative to group housed animals, and rats isolated for three weeks exhibited a more anxiogenic behavioural profile in the light-dark box compared to socially housed rats (Morinan, Parker, Rich, Cariuk & Horton, 1992). It has been suggested that significant alterations in central neurochemical activity may parallel some of the behavioural effects seen in isolated animals (Oehler, Jahkel & Schmidt, 1987). For example, isolated rats showed reduced mesocortical DA turnover (Blanc, et al., 1980) and demonstrated post synaptic 5-HT receptor supersensitivity (Wright, Ismail, Upton & Marsden, 1991) relative to group housed animals. Taken together, it would appear that individually housed mice may be behaviourally and neurochemically vulnerable to the influences of anxiogenic stimuli and/or paradigms which produce such effects.
In addition to the standard protocol in this laboratory of housing animals individually, surgical intervention is also routinely necessary. Indeed, pharmacological challenges are frequently administered centrally and it should be considered that the intraventricular or intracerebral implantation of a cannula may influence subsequent physiological and/or behavioural data. For example, the major stress associated with surgical intervention has been demonstrated to increase peripheral catecholamine levels in nonhuman primates (Udelsman, Goldstein, Loriaux & Chrousos, 1987), to produce an analgesic effect in mice (Jayaram, Singh & Carp. 1995) and, in some instances, to provoke psychiatric complications in human subjects (Nader et al., 1990). Accordingly, it should be considered the that imposition of surgical stress on individually housed mice may influence subsequent behavioural and neurochemical reactivity in paradigms designed to assess behavioural pathology and anxiety, in particular.

Experiment 2 examined (a) the influence of surgical stress on basal levels of anxiety in the light-dark box in singly housed CD-1 mice and (b) the induction of anxiety in these animals following exposure to uncontrollable footshock. Surgical stress was operationally defined as the cumulative surgical experience, including intraperitoneal administration of the anaesthetic, scalp cleansing and incision, implantation of the cannula, as well as the particulars associated with wound closure.

**Method**

**Subjects**

Twenty eight naive, male, CD-1 mice were obtained from Charles River Canada (St. Constant, Quebec) at five weeks of age. Animals were acclimatized to the animal
facility for approximately 2 months prior to the Experiment. All mice were maintained on a 12 hour light-dark cycle (lights on 7:00 a.m.). Food and water were available ad libitum. One half of the mice were individually housed for 10 days prior to assessment in the light-dark box, while the remaining animals were implanted with a cannula in the lateral ventricle and then permitted a 10 day recovery period, prior to testing in the light-dark box.

**Surgery**

Surgical anesthesia was induced by an intraperitoneal injection of Somnotol (sodium pentobarbitol). The mouse was placed in a David Kopf Stereotaxic instrument fitted with a mouse adapter, its scalp cleansed and an anterior to posterior incision was made to expose the skull surface. Coordinates used for the surgical implantation of a 23 gauge cannula into the lateral ventricle were A.P. +0.8 mm. from Bregma, 0.7 mm. lateral from the midline and ventral -2.8 mm. from a flat skull surface. A 30 gauge stylette was employed to increase the patency of the implanted cannula. Following surgery, animals were housed individually, placed on a warm heating pad and provided a dietary supplement (Meritene nutritional supplement in wet crushed food pellets) in a recovery room for a minimum of three days. Once recovered, mice were returned to the main animal holding area for approximately 7 days until the commencement of behavioural testing.

**Apparatus**

The light-dark test box and the shock apparatus were identical to those described in Experiment 1.
Procedure

All subjects were tested in the light-dark box for 10 minutes on each of three consecutive days between 9:00 a.m. and 12:00 p.m. Each mouse was taken from its home cage and placed in the center of the light chamber facing the opening in the partition to the dark section of the box. The latency for the mouse to enter the dark chamber, the number of transitions between chambers and the amount of time spent in each section was recorded during each session. An animal was considered to have entered a chamber and timing commenced only when all four paws were positioned in that chamber.

Subsequent to baseline anxiety assessment in the light-dark paradigm, half of the mice in each of the two groups (mice exposed to cannulation procedure and non-cannulated mice) were assigned to an acute uncontrollable footshock condition, while the remaining animals were assigned to a no footshock condition. On the immediate test day, animals received either 60 uncontrollable footshocks (150 µA, 60 x 6 sec duration, with a 59 sec inter-trial interval) or were exposed to the shock apparatus for an identical period of time (66 min.), but the stressor was withheld. Immediately following exposure to the stressor, subjects were tested in the light-dark box according to the procedure outlined earlier. A final test was conducted in the apparatus one week post-stressor.

Results

Baseline Data: AI scores were analyzed over three consecutive Baseline days to determine if the animals in each surgical condition (i.e., exposed to surgical stress or not exposed to surgical stress) differed on AI scores in the light-dark apparatus prior to stressor exposure. The analysis of variance of the AI scores on the three Baseline days
revealed a significant Day by Condition interaction prior to shock treatment ($F_{(2,52)} = 5.57$, $p < 0.01$ for the Day x Condition interaction). Subsequent simple effects analysis indicated the mice exposed to surgical stress were significantly more anxious (i.e., lower AI scores) than mice not exposed to surgical stress on Day 1, ($F_{(1,29)} = 6.46$, $p < 0.05$), Day 2 ($F_{(1,26)} = 16.44$, $p < 0.001$) and on Day 3 ($F_{(1,38)} = 17.77$, $p < 0.001$) of the Baseline procedure (see Figure 3a)

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Insert Figure 3a about here

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Post Treatment Data: An analysis of variance of the AI scores from the immediate and one week post stressor tests among animals from each of the two surgical conditions (i.e., surgical cannulation or no surgical cannulation) exposed to No shock or 60 shocks was conducted. Although the analysis failed to reveal a significant Day x Condition x Shock interaction, a significant Day x Shock interaction ($F_{(1,24)} = 26.45$, $p < 0.01$) was detected. Subsequent simple effects analysis indicated that the AI levels of mice exposed to uncontrollable footshock were significantly lower (i.e., more anxiogenic) than AI scores of mice exposed to No Shock on the immediate test day ($F_{(1,24)} = 18.47$, $p < 0.001$). Anxiogenic effects were restricted to the immediate post stressor interval as the Shock and No shock groups were indistinguishable from one another on AI scores one week following initial stressor exposure (see Figure 3b). A significant main effect of Condition in the post stressor analysis ($F_{(1,24)} = 8.08$, $p < 0.01$) revealed the cannulated animals were
Figure 3a  (Upper graph) Mean (± S.E.M) anxiety index (Time/sec /Transition) on 3 consecutive Baseline days of cannulated and non cannulated CD-1 mice tested for 10 minute sessions in the light-dark apparatus (n=14/group)

Figure 3b  (Lower graph) Mean (± S.E.M) anxiety index (Time/sec /Transition) of CD-1 mice exposed to an acute session of 60 uncontrollable footshocks or No shock and assessed in the light-dark box immediately and one week following stressor exposure (n=14/group). Data are collapsed across surgical condition
significantly more anxious than the non-cannulated mice (indicated by lower AI levels) independent of the day of testing or stressor exposure

Insert Figure 3b about here

**Discussion**

Isolation of animals has been reported to provoke various behavioural disturbances including neophobia (Parker & Morinan, 1986), aggression (Rodgers & Cole, 1993b; Wilmot, Fico, Vanderwende & Spoerlein, 1989), hyperactivity in novel environments (Gentsch, Lichtsteiner, Frischknecht, Feer & Siegfried, 1988) and anxiety (Stanford, Parker & Morinan, 1988, Vasar et al., 1993) For instance, Maisonnette et al. (1993) reported that rats isolated for two hours displayed decreased open arm entries and time in the open arms of the elevated plus maze relative to socially housed animals. Although many laboratories have replicated these data (Vasar et al., 1993, Wright, Ismail, Upton & Marsden, 1990), others failed to detect significant differences in anxiety between isolated and group housed animals in the elevated plus maze (Rodgers & Cole, 1993b) and Hilakivi, Ota and Lister (1989) reported that isolation actually increased open arm entries. Isolated rats also exhibited an anxiogenic profile in the light dark box, (i.e., significantly less time spent in the light and fewer transitions made between the light and dark compartments) compared to socially reared rats (Morinan et al., 1992; Sanford et al., 1988). It is noteworthy that social isolation may also lead to neurochemical alterations affecting DA (Jones, Hernandez, Kendall, Marsden & Robbins, 1992), NE (Heritch,
Henderson & Westfall, 1990), 5-HT (Crespi, Wright & Mobius, 1992) and CCK (Vasar et al., 1993) systems and these changes may give rise to a wide variety of the reported behavioural variations.

In view of the behavioural and neurochemical consequences of isolation, it should be considered that individually housed animals may be particularly vulnerable to, or less able to cope with, subsequent stressor imposition (Morinan et al., 1992). In fact, isolation of animals altered the frequency of attempts to escape from footshock (Nishikawa & Tanaka, 1978), increased startle reactivity (Viken, Moore, Knutson & Fordyce, 1982) during footshock and influenced the magnitude of shuttle-escape deficits seen following uncontrollable footshock (Anisman & Sklar, 1981). Moreover, individual housing influenced the activity of mesocortical DA in response to footshock (Blanc et al., 1980) and enhanced the vulnerability of rats to stressor and amphetamine induced behavioural sensitization (Ahmed, Stinus, LeMoal & Cador, 1995).

It is now well established that surgical procedures may also provoke a stress response (Jayaram, et al., 1995; Naber et al., 1990; Pollock, Lotzova & Stanford, 1991). For example, surgical manipulation stimulates the hypothalamic-pituitary-adrenal (HPA) axis (i.e., CRH, ACTH release), and the sympathetic nervous system (Udelsman et al., 1987), and has been reported to induce the expression of heat shock proteins (cellular stress response genes) in parallel with HPA activation (Udelsman, Blake & Holbrook, 1991), to suppress various aspects of the immune response in animals (Freire-Garabal, Belmonte, Balboa & Nunez, 1992) and to impair natural killer cell cytotoxicity in humans (Pollock et al., 1991).
In view of the observation that individually housed animals may be rendered vulnerable to stressor exposure, it should be considered that the stress of surgical intervention may influence the behavioural and/or neurochemical response profile of isolated animals. It was of interest therefore, to compare the basal anxiety levels, as well as AI alterations provoked by footshock, of individually housed mice which had been exposed to a surgical cannulation procedure to those of individually housed, non surgically stressed animals. On each of three consecutive days of baseline testing in the light dark box, cannulated mice in Experiment 2, exhibited significantly greater anxiety (as revealed by decreased AI scores) than non cannulated animals. It is important to consider that if the surgical cannulation procedure, superimposed upon isolated animals, influenced the behavioural expression of anxiety in the light dark apparatus, it may also have neurochemical and behavioural consequences in several other paradigms. Such a prospect has important implications for investigations employing any type of surgical procedure and deserves further investigation.

It is significant that although the AI levels of the mice in the cannulated and non-cannulated groups did not decrease over the three days of baseline testing, the total time in the light compartment and the transition frequency for each of the groups declined across the three test days. Moreover, the decreases in these two behavioural measures with repeated testing were most extreme for the cannulated mice. It should be taken into account that any detection of anxiety enhancement, due to environmental or pharmacological challenge following the baseline procedure, would be compromised by these low time and transition baseline measures (see also Rodgers & Cole, 1993). On the
basis of such possible floor effects with repeated testing in the light dark apparatus, it was concluded that a three day baseline protocol would be omitted in any subsequent experiments employing isolated, surgically cannulated mice (i.e., Experiments 3 and 4).

In accordance with results of Experiment 1a, exposure of animals to 60 uncontrollable footshocks provoked significant increases in anxiety, as indicated by decreases in the AI. Considering the enhanced sensitivity of the cannulated animals to the anxiety provoking stimuli in the light dark box (i.e., in the Baseline assessment), it was of interest to ascertain if cannulated animals might be more vulnerable to the effects of footshock than non-cannulated mice. No significant differences in AI scores were detected between the cannulated and non-cannulated mice immediately following footshock, however. It should be noted that, although the stressor provoked decrements in the AI levels of the two groups were similar in magnitude, the significant differences in the AI between cannulated and non-cannulated mice prior to stressor imposition preclude any conclusions regarding differential stressor sensitivity. It is conceivable that the animals exposed to the surgical cannulation procedure were more vulnerable to subsequent stressor exposure, but such a distinction could not be detected in the current paradigm employed. Such a differentiation might be better explored employing both behavioural and neurochemical indices, with particular attention focused on the concordance of pre-stressor basal levels.

Taken together, the data suggest the behavioural expression of anxiety is not only influenced by exposure to aversive procedures (e.g., footshock, immobilization), but also by experiential factors which encompass the life of the organism. For example, social
isolation of animals (i.e., individual housing) may provoke various behavioural and neurochemical alterations which render animals vulnerable to further stressful manipulations. The present investigation revealed that a history of surgical stress did, indeed influence anxiety levels of isolated CD-1 mice in the light dark box.
EXPERIMENTS 3A - 3C

It has been well documented that systemic administration of CCK-4 among healthy volunteers provokes panic attacks reminiscent of those evident in panic patients (Bradwejn & Koszycki, 1994), while peripheral or intracerebral administration of CCK and CCK agonists induces anxiogenic behaviour in animal models of anxiety (Harro et al, 1990c; Harro & Vasar, 1991b; Singh, Field, Hughes, Vass & Woodruff, 1991). The purpose of Experiment 3 was to (a) evaluate the influence of intraventricular administration of varying doses of CCK-8S on behaviour of the CD-1 mouse in the light-dark box model of anxiety and (b) determine whether re-exposure to CCK-8S might exacerbate anxiogenic behaviour.

Method

Subjects

One hundred and eight naive, male, CD-1 mice were obtained from Charles River Canada (St. Constant, Quebec) at five weeks of age. Animals were acclimatized to the animal facility until an acceptable age and/or weight for surgical manipulation (3 months old or 30 g) was achieved. All mice were housed four per cage and maintained on a 12 hour light-dark cycle. Food and water were available ad libitum.

Drug

Sulfated cholecystokinin (CCK-8S), obtained in 0.25 mg quantities from Sigma Chemicals was dissolved in 15 μl of a 1 M sodium bicarbonate (NaHCO₃) solution. The appropriate quantities of CCK-8S solution and 0.9% sterile saline were added to individual drug vials to obtain the following desired concentrations of drug in a 1μl
volume: 50 ng CCK-8S, 25 ng CCK-8S and 5 ng CCK-8S, 2.5 ng CCK-8S and 0.5 ng CCK-8S. The vehicle solution consisted of sodium bicarbonate and saline

Procedure

Details of the surgical implantation of the cannulae are identical to those described in Experiment 2. Following recovery from surgery, animals were assigned to either Experiment 3a, 3b or 3c. Mice were subsequently assigned to one of 6 treatments within each Experiment (n = 6-7 per group). Cholecystokinin or vehicle was administered intraventricularly, employing a Hamilton 5 μl syringe connected to a 30 gauge injector. A 1 μl volume was injected over a one minute period and the injector was left in position for an additional minute to ensure adequate drug diffusion. The stylette was then replaced. All subjects received an Initial intraventricular injection followed by a Challenge injection, 7 days later.

In Experiment 3a, half of the mice received a 5 ng CCK-8S injection on the first test day and were tested in the light-dark box (see Experiment 1) 15 minutes following central drug administration. One week following initial CCK-8S administration, one-third of these mice received the same dose of CCK-8S (5 ng), one-third received one-tenth of the initial dose of CCK-8S (0.5 ng), while the remaining third were injected with vehicle. The remaining half of the subjects were injected with vehicle on the first test day and challenged with 5 ng CCK-8S, 0.5 ng CCK-8S, or vehicle one week later. In all instances, mice were tested over a 10 minute test interval in the light-dark box 15 minutes following drug administration.
In Experiment 3b, half of the animals were administered 25 ng CCK-8S on the first test day, while the remaining half received vehicle 15 minutes prior to testing. One week following initial drug administration, one third of the mice within each of those groups was challenged with 25 ng CCK-8S, one-third with 2 5 ng CCK-8S and one-third with vehicle.

In Experiment 3c, the identical procedure was followed employing a dose of either 50 ng CCK-8S or vehicle for the initial test day and one of 50 ng CCK-8S, 5 ng CCK-8S or vehicle on the second test day.

Following completion of the Experiments, animals were overdosed with sodium pentobarbital and perfused intracardially with 0.9% saline followed by a 10% formalin solution. Brains were removed from the skull, blocked, frozen and sectioned at 40μ. Coronal brain sections were stained with cresyl violet and examined under a microscope to verify cannula placement in the lateral ventricle.

Results

Microscopic examination of brain sections of mice included in Experiments 3a, 3b and 3c confirmed accurate cannulae placements within the lateral ventricle. Moreover, intraventricular injections did not result in any observable tissue damage.

Experiment 3a

An analysis of variance of the AI scores of mice administered 5 ng CCK-8S or vehicle on the first test day (Initial Dose) and 5 ng CCK-8S, 0.5 ng CCK-8S or vehicle one week later (Challenge Dose) failed to reveal a significant Initial Dose x Challenge Dose x Day interaction ($F_{(2,31)} = 0.69, p>.05$) or a Challenge Dose x Day interaction ($F_{(2,31)}$
A significant Initial Dose x Day interaction was detected ($F_{(1,11)} = 7.37$, $p<05$). However, simple effects analysis revealed a lack of a significant Dose effect on either of the test days (see Fig. 4a & 4b).

Insert Figure 4a & 4b about here

Experiment 3b

An analysis of variance of the AI scores of mice administered 25 ng CCK-8S or vehicle on the first test day (Initial Dose) and 25 ng CCK-8S, 2.5 ng CCK-8S or vehicle one week later (Challenge Dose) failed to reveal a significant Initial Dose x Challenge Dose x Day interaction ($F_{(2,32)} = 0.04$, $p>05$), or a significant Initial Dose x Day interaction ($F_{(1,32)} = 0.29$, $p>05$) (see Fig. 5a). However, a significant Challenge Dose x Day interaction ($F_{(2,32)} = 3.30$, $p<05$) was detected. Simple effects analysis and Newman Keuls multiple comparisons ($\alpha=0.05$) revealed that, mice challenged with 25 ng CCK-8S intraventricularly were significantly more anxious (i.e., lower AI) in the light dark paradigm relative to mice administered either 2.5 ng CCK-8S or vehicle ($F_{(2,32)} = 5.36$, $p<01$). These behavioural effects were evident despite the observation that the 25 ng dose of CCK-8S was ineffective in increasing anxiety when it was first administered, on the initial test day (see Figure 5b).

Insert Figure 5a & 5b about here
Figure 4a. (Upper graph) Mean (± S E M ) anxiety index (Time/sec./Transition) of CD-1 mice administered either 5 ng CCK-8S or vehicle (Initial Dose) and tested in the light-dark box 15 minutes following central drug administration (n=18-19/group). Behavioural tests were 10 minutes in duration.

Figure 4b. (Lower graph) Mean (± S E M ) anxiety index (Time/sec./Transition) of CD-1 mice administered either vehicle, 0.5 ng CCK-8S or 5 ng CCK-8S (Challenge Dose) one week following Initial Drug exposure. Mice were tested in the light-dark box 15 minutes following central drug administration (n=6-7/group). Behavioural tests were 10 minutes in duration.
Figure 5a: (Upper graph) Mean (± S.E.M.) anxiety index (Time/sec/Transition) of CD-1 mice administered either 25 ng CCK-8S or vehicle (Initial Dose) and tested in the light-dark box 15 minutes following central drug administration (n=18-20/group). Behavioural tests were 10 minutes in duration.

Figure 5b: (Lower graph) Mean (± S.E.M.) anxiety index (Time/sec/Transition) of CD-1 mice administered vehicle, 2.5 ng CCK-8S or 25 ng CCK-8S (Challenge Dose) one week following Initial Drug exposure. Mice were tested in the light-dark box 15 minutes following central drug administration (n=6-7/group). Behavioural tests were 10 minutes in duration.
Experiment 3c

An analysis of variance of the AI scores of mice administered 50 ng CCK-8S or vehicle on the first test day (Initial Dose) and challenged with 50 ng CCK-8S, 5 ng CCK-8S or vehicle one week later (Challenge Dose) failed to reveal a significant Initial Dose x Challenge Dose x Day interaction ($F_{(2,31)} = 0.77$, $p > 0.05$), thereby indicating that prior administration of 50 ng CCK-8S did not lead to an exacerbation of anxiogenic behaviour in the light dark box upon challenge one week later. However, a significant Initial Dose x Day interaction ($F_{(1,31)} = 5.37$, $p < 0.05$) and a significant Challenge Dose x Day interaction ($F_{(2,31)} = 3.75$, $p < 0.05$) were revealed. Subsequent simple effects analysis indicated that, on the first day of testing, the AI scores of mice administered 50 ng CCK-8S were significantly lower than those of mice administered vehicle ($F_{(1,31)} = 13.63$, $p < 0.01$) (see Figure 6a). This behavioural effect was restricted to the immediate post drug administration interval. In addition, simple effects analysis and Newman Keuls multiple comparisons revealed that, mice challenged with 50 ng CCK-8S one week later were significantly more anxious (i.e., lower AI) in the light dark box relative to mice challenged with 5 ng CCK-8S or vehicle ($F_{(2,31)} = 6.05$, $p < 0.01$) regardless of the drug administered one week previous (see Figure 6b)

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Insert Figure 6a and 6b about here

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Figure 6a (Upper graph) Mean (± S.E.M) anxiety index (Time/sec/Transition) of CD-1 mice administered 50 ng CCK-8S or vehicle (Initial Dose) and tested in the light-dark box 15 minutes following central drug administration (n=18-19/group) Behavioural test were 10 minutes in duration.

Figure 6b (Lower graph) Mean (± S.E.M) anxiety index (Time/sec/Transition) of CD-1 mice administered vehicle, 5 ng CCK-8S or 50 ng CCK-8S (Challenge Dose) one week following Initial Drug exposure. Mice were tested in the light-dark box 15 minutes following central drug administration (n=6-7/group) Behavioural tests were 10 minutes in duration.
Discussion

The data of Experiment 3 are consistent with investigations in other laboratories which have detected anxiogenic effects in the elevated plus maze (Harro et al., 1990c) and light dark models of anxiety (Singh et al., 1991a) following systemic administration CCK-8 (Chopin & Briley, 1993), CCK-4 (Rex, Barth, Voigt, Domeney & Fink, 1994), or related CCK receptor agonists (Singh et al., 1991a & b). However, there remains some controversy in the literature regarding which CCK receptor agonists, in particular, are most effective in the provocation of anxiety. For example, while some investigators have reported an anxiogenic profile in the light dark box and elevated plus maze in rats following systemic administration of the selective CCKB receptor agonist, CCK-4, but not following the CCKAB receptor agonist, CCK-8S (Rex et al., 1994a), others have demonstrated increased anxiety in rats and mice following systemic or intraventricular administration of CCK-8 receptor agonists, such as caerulein in the same paradigms (Harro et al., 1990c; Singh et al., 1991b). It should be considered that these discrepant data may have been influenced by a number of variables, including species and/or paradigm differences, the specific agonists employed, as well as the route of drug administration, among others. In view of evidence to date implicating central CCKH receptors in the provocation of anxiety (Derrien, McCort-Tranchepain, Ducos, Roques & Durieux, 1994; Rex et al., 1994, Singh et al., 1991a & b, but cf. Hendrie et al., 1993) and the demonstration that CCK-8S activates central CCKH receptors, the present results documenting anxiogenic effects in the light dark paradigm following intraventricular administration of CCK-8S were not surprising
Central accessibility of CCK-8S is obviously not at issue in describing behavioural change in the present series of Experiments since the peptide was administered intraventricularly. However, it should be underscored that central accessibility of peripherally administered CCK may be determined by the ability of the peptide to cross the blood brain barrier (Albus, 1988; Crawley, 1985). Harro, Vasar & Bradwejn (1993) suggested that systemically administered CCK may provoke behavioural change by influencing central CCK receptors in regions not fully protected by the blood brain barrier (i.e., nucleus tractus solitarius and area postrema of the brainstem). While this is an interesting speculation, and the detection of anxiogenic effects following peripheral CCK administration suggests central CCK\textsubscript{B} receptor activation (Harro & Vasar, 1991a), the validity of this hypothesis has not yet been established. However, it should be considered that activation of peripheral CCK\textsubscript{A} receptors following systemic administration of CCK agonists may complicate data interpretation and have confounding effects on satiety (Dourish et al., 1989) and sedation (Harro & Vasar, 1991b). In investigations of anxiogenesis, it therefore seems judicious to administer CCK peptide fragments centrally.

Crawley (1985) has recommended CCK doses less than 100 ng when intraventricular routes of administration are employed and 10 ng or less when CCK is administered intracerebrally to effect behavioural change. The CD-1 mice in the present series of Experiments received intraventricular CCK-8S doses within the suggested range for central CCK\textsubscript{B} receptor activation (i.e., 5 ng, 25 ng or 50 ng). The highest dose of CCK-8S employed, 50 ng, provoked a significant anxiogenic response in CD-1 mice, the 25 ng dose effected only a small increase in anxiety, while the dose of 5 ng CCK-8S had
no effect on behaviour in the light dark paradigm. Thus, it appears that when CCK-8S is administered intraventricularly in the CD-1 mouse, the threshold for central CCKR receptor activation and subsequent behavioural change in the light dark paradigm is reached with approximately 50 ng CCK-8S.

Surprisingly, there are currently no data available describing the anxiogenic effects of intraventricular CCK-8S administration in the light dark paradigm in mice. Cholecystokinin-8 and CCK-4 are typically administered peripherally and central administration has been ordinarily restricted to the nucleus accumbens and VTA in rats. Studies in which CCK has been administered intracerebrally have been conducted in the investigation of the functional relationship between CCK and DA turnover in the mesolimbic system however, and not in the assessment of anxiety (Crawley, 1991, 1994; Vaccarino, 1994). Accordingly, the results of Experiment 3 contribute significant data to the literature which is deficient with respect to the behavioural consequences of central CCK-8S administration on anxiety.

In addition to establishing an anxiogenic profile in the light-dark box following CCK-8S administration, it was also of interest to determine if prior central CCK-8S administration would provoke behavioural sensitization to subsequently applied CCK. Accordingly, animals were challenged with the original dose or one-tenth of the original dose of CCK-8S one week after the initial administration of the anxiogenic agent. Re-exposure to a lower (i.e., one-tenth of initial) challenge dose of CCK did not enhance behavioural effects in the light-dark box relative to that observed on the initial drug trial. In effect, behavioural sensitization was not detected with the lower doses of CCK-8S.
employed and the one week inter-drug interval. Nevertheless, some interesting behavioural effects appeared following re-exposure to the initial dose of CCK-8S. For example, in Experiment 3a, challenge with 5 ng CCK-8S failed to provoke anxiogenic behaviour, regardless of prior drug experience. However, in Experiment 3b, a different response profile emerged. The 25 ng challenge dose of CCK-8S augmented anxiety in the light dark paradigm relative to mice which were challenged with vehicle or 2.5 ng CCK-8S, despite the observation that 25 ng CCK-8S did not provoke a significant anxiogenic response one week previously. Moreover, the anxiogenic behaviour of mice challenged with 25 ng CCK-8S was comparable irrespective of prior drug treatment (i.e., vehicle or 25 ng CCK-8S initially). In effect, the 25 ng dose of CCK-8S was sufficient to increase anxiety in mice if these animals were either exposed to the light dark paradigm or treated with 25 ng of the anxiogenic one week earlier. Taken together, it seems that anxiogenic behaviour following challenge with 25 ng CCK-8S was determined by an interaction between the challenge dose of CCK-8S and prior experience in the light dark paradigm one week previously. It is noteworthy that these behavioural effects appeared as early as one week following initial drug/paradigm exposure. One could speculate that, given a longer interval between initial and challenge testing or the administration of a slightly higher dose of CCK-8S, drug induced sensitization may have been demonstrated following challenge, such that mice may have exhibited significantly greater anxiety upon re-exposure to CCK-8S than following initial exposure.

Intraventricular challenge with 50 ng CCK-8S in Experiment 3c also provoked anxiogenic behaviour relative to challenge with either 5 ng CCK-8S or vehicle and such
behavioural effects were independent of initial drug exposure. Although these data are not surprising, given the anxiogenic profile following the initial administration of 50 ng CCK-8S, it should be noted that, upon challenge with 50 ng, mice which received 50 ng initially had a tendency to be more anxious than animals which received vehicle one week previous. It should be emphasized however, that anxiety levels of these two groups did not differ significantly upon challenge under the present test conditions, but it is conceivable that a longer interval between successive drug exposures may have been required to effect significant behavioural differences.

Although only certain doses of CCK-8S provoked anxiogenic behaviour in the light dark box, it is likely that any exposure to CCK-8S induced alterations in central neurochemical activity (e.g. GABA, NE, DA, 5-HT). Indeed, central CCK-8S administration may have given rise to central neurochemical variations, but these variations may not have been expressed immediately as an increase in anxious behaviour in the light dark paradigm. The data of the present Experiment suggest that the behavioural consequences of antecedent neurochemical change may only become apparent upon challenge at a later date and may also be influenced by prior experience in the paradigm. It remains to be determined, however, whether such effects are unique to this particular paradigm under investigation.

In addition to the dose of drug administered and prior experience of the organism, the interval between stimulus presentations is also a critical element in the development of sensitization and is important in determining subsequent behaviour (Post, 1980). While the interval between successive stimulations can vary greatly in the induction of sensitization,
from one day or less, to several months, behavioural and/or neurochemical sensitization can often be demonstrated in seven to ten days (for reviews see Antleman, 1988; Robinson, 1988). Accordingly, the choice of a one week interval between initial and challenge drug administration was considered appropriate in the present series of Experiments. However, it would also have been of interest to follow these animals for several additional weeks to determine the permanence and/or growth of any increased behavioural responsiveness to intraventricular CCK-8S administration. In addition, it should be considered that the dose of CCK-8S administered initially in the present series of Experiments may have interacted with the chosen interval between initial and challenge exposures such that higher doses may have induced behavioural sensitization in a shorter period than a lower dose. Similarly, it remains to be determined whether sensitization would have been evident following the administration of lower challenge doses (i.e., one-tenth of the initial dose) if the interval between successive anxiogenic drug exposures had been protracted.

In summary, intraventricular CCK-8S administration in CD-1 mice produced anxiogenic behaviour in the light dark box model of anxiety which was dose dependent. Upon initial exposure to CCK-8S, a dose of 50 ng was required to significantly alter scores of time in light and transitions into light. Sensitization was not observed among animals challenged one week later with one-tenth of the dose administered initially. Moreover, re-exposure of mice to the initial dose of 5 ng CCK-8S did not alter behaviour in the light dark box. However, challenge with 25 ng CCK-8S one week subsequent to initial testing did provoke anxiogenic consequences which appeared to be contingent upon
prior experience in the light dark paradigm. Anxiety was also detected following challenge with 50 ng CCK-8S and this effect was more prominent in animals which received 50 ng CCK-8S one week earlier than in mice which received vehicle. Taken together, the provocation of anxiogenic behaviour in the light dark paradigm following intraventricular CCK-8S administration is not only dose dependent, but also appears to be influenced by prior experience of the organism and the particulars of the testing procedure. Moreover, it is likely that CCK-8S interfaces with multiple central neurochemical systems in the promotion of anxiety and these interactions may lead to the induction of sensitization.
EXPERIMENTS 4A AND 4B

Available data to date suggest acute exposure to a stressor may have long term behavioural and/or neurochemical consequences (Anisman & Zacharko, 1990; Robinson, 1988). In effect, initial encounters with stressful events, which may result in the sensitization of central neurochemical activity, may have a significant influence on an organism's vulnerability to subsequent behavioural and/or psychological disturbances (Zacharko & Anisman, 1989). Accordingly, it should be considered that sensitization and cross-sensitization may contribute to the development and/or exacerbation of a number of disorders, including anxiety and panic (Antelman, 1988; Zacharko et al., 1995).

In view of the observation that stressor exposure and CCK-8S administration can induce anxious behaviour (Experiment 1 and 3), the purpose of Experiment 4 was to determine if behavioural cross-sensitization between stressors (footshock or restraint) and CCK-8S would favour exacerbated anxiogenic reactivity in the light-dark box paradigm in CD-1 mice. In particular, Experiment 4a assessed whether previous administration of CCK-8S affected anxiogenic behaviour following exposure to a mild stressor one week later, while Experiment 4b examined whether prior stressor exposure influenced behaviour in the light-dark box after subsequent challenge with a low dose of CCK-8S.

Method

Subjects

Ninety-one naive, male, CD-1 mice were obtained from Charles River Canada (St. Constant, Quebec) at five weeks of age. Animals were acclimatized to the animal facility until an acceptable age and/or weight for surgical manipulation was achieved (3 months
old or 30 g). All mice were housed four per cage and maintained on a 12 hour light-dark cycle. Food and water were available ad libitum.

**Apparatus**

The light-dark test box, uncontrollable footshock apparatus, and restraint tubes were identical to those described in Experiment 1.

**Drug**

Sulfated CCK-8S, obtained in 0.25 mg quantities from Sigma Chemicals was dissolved in 15 μl of a 1 M sodium bicarbonate (NaHCO₃) solution. The appropriate quantities of CCK-8S solution and 0.9% sterile saline were then added to individual drug vials to obtain the following desired concentrations of drug: 50 ng CCK-8S or 5 ng CCK-8S per 1 μl injection. The vehicle solution consisted of sodium bicarbonate and sterile saline.

**Procedure**

Details of the surgical implantation of the cannulae are identical to those described in Experiment 2. Following recovery from surgery, animals were assigned to one of two treatment conditions. Half of the subjects were exposed to an acute stressor followed by either CCK-8S or vehicle challenge 7 days later (n = 7-8/group) (Experiment 4a), while the remaining animals were administered CCK-8S or vehicle initially and subjected to either stress or no stress conditions 7 days later (n = 7-8/group) (Experiment 4b). Each part of the experiment was comprised of 6 groups. Specifics of the treatment parameters follow.
In Experiment 4a, mice received either 50 ng CCK-8S or vehicle, intraventricularly, in a 1 µl volume, 15 minutes prior to behavioural testing in the light-dark box. Intraventricular drug injection was accomplished employing a Hamilton 5 µl syringe connected to a 30 gauge injector. A 1 µl volume was injected over a one minute period and the injector was left in position for an additional minute to ensure adequate drug diffusion. The stylette was then replaced. One week later, one-third of the animals within each group was placed in a restraint tube for 15 minutes, one-third of the animals received 2 uncontrollable footshocks, while the remaining animals comprised the no stressor condition. Subjects were evaluated in the light-dark box immediately following stressor treatment.

In Experiment 4b, mice were exposed to either 30 minutes of restraint, 30 uncontrollable footshocks or to a no treatment condition and were tested in the light-dark box immediately thereafter. Seven days later, the mice of each of the stressor conditions were subdivided such that half of the animals were challenged with 5 ng CCK-8S and half were challenged with vehicle. Subjects were tested in the light-dark box 15 minutes following drug or vehicle administration.

Following completion of Experiments 4a and 4b, animals were overdosed with sodium pentobarbital and perfused intracardially with 0.9% saline followed by a 10% formalin solution. Brains were removed from the skull, blocked, frozen and sectioned at 40μ. Coronal brain sections were stained with cresyl violet and examined under a microscope to verify cannula placement in the lateral ventricle.
**Results**

Microscopic examination of brain sections of mice included in Experiments 4a and 4b confirmed accurate cannulae placements within the lateral ventricle. Moreover, intraventricular injections did not result in any observable tissue damage.

**Experiment 4a**

An analysis of variance of the AI scores of mice administered 50 ng CCK-8S or vehicle on the first test day (Drug Condition) and exposed to 15 minutes of restraint, 2 uncontrollable footshocks or a no stress condition (Stress Condition) one week later failed to reveal a significant Drug x Stress x Day interaction ($F_{(2,38)} = 0.01, p > 05$). A significant Drug x Day interaction was detected, however ($F_{(1,38)} = 10.58, p < 01$). Simple effects analysis indicated animals administered 50 ng CCK-8S on the first test day were significantly more anxious when tested in the light dark box than mice injected with vehicle ($F_{(1,38)} = 14.43, p < 0.01$) (see Figure 7a). In addition, a significant Stress x Day interaction was also detected ($F_{(2,38)} = 3.79$, $p < 05$). Simple effects analysis and Newman Keuls multiple comparisons revealed that on the second test day, one week following administration of either 50 ng CCK-8S or vehicle, mice exposed to two uncontrollable footshocks were significantly more anxious than non-stressed mice. Animals restrained for 15 minutes did not differ significantly from shocked or non-stressed mice on the second test day (see Figure 7b).

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Insert Figure 7a & 7b about here

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**Figure 7a** (Upper graph) Mean (± S.E.M) anxiety index (Time/sec/Transition) of CD-1 mice administered 50 ng CCK-8S or vehicle (Drug Condition) and tested in the light-dark box 15 minutes following central drug administration (n=22-24/group). Behavioural tests were 10 minutes in duration.

**Figure 7b** (Lower graph) Mean (± S.E.M) anxiety index (Time/sec/Transition) of CD-1 mice exposed to a no stress condition (NS), 15 minutes of restraint stress (RS), or 2 uncontrollable footshocks (FS) one week following the administration of vehicle or 50 ng CCK-8S (n=7-8/group). Behavioural tests were 10 minutes in duration.
Experiment 4b

An analysis of variance of the AI scores of mice exposed to 30 minutes of restraint, 30 uncontrollable footshocks or to a no stressor condition on the first test day (Stress Condition) and challenged with 5 ng CCK-8S or vehicle one week later (Drug Challenge Condition) revealed a significant Stress x Drug x Day interaction ($F_{1,40} = 4.20$, $p<.05$), indicating that prior stressor exposure had an effect on anxiogenic behavior following challenge one week later in the light dark box. Subsequent simple effects analysis and Newman Keuls multiple comparisons revealed that animals exposed to 30 minutes of restraint and injected with 5 ng CCK-8S one week later were significantly more anxious (lower AI) on the second test day than mice in the 30 minute restraint - vehicle, no stress - vehicle, no stress - 5 ng CCK-8S, and 30 uncontrollable footshocks - vehicle conditions ($F_{1,40} = 3.79$, $p<.05$). Mice in the 30 footshock - 5 ng CCK-8S condition did not differ significantly on AI from animals in any of the other groups on the second test day (see Figure 8b).

In addition to the significant three-way interaction, a significant Stress x Day interaction was revealed ($F_{1,40} = 4.43$, $p<.05$). Subsequent simple main effects analysis and Newman Keuls multiple comparisons indicated that, on the first test day, the AI levels of mice exposed to 30 minutes of restraint and 30 uncontrollable footshocks were significantly lower than the AI levels of animals in the no stress condition ($F_{1,40} = 10.28$, $p<.001$), but the AI levels of animals exposed to restraint did not differ from those of mice exposed to footshock (see Figure 8a). The analysis of variance also revealed a significant Dose x Day interaction ($F_{1,40} = 12.98$, $p<.001$). Simple main effects analysis indicated
animals administered 5 ng CCK-8S were significantly more anxious in the light dark box on the second test day than mice that received vehicle \((F_{1,24} = 9.34, p < 0.01)\)

Insert Figure 8a & 8b about here

Discussion

It will be recalled that a single, prior exposure to a drug or stressful event can influence subsequent responsivity to a drug or stressor, such that a long-lasting hypersensitivity, or sensitization to a challenge stimulus may be produced (Antelman & Caggiula, 1990). Moreover, it has been suggested that sensitization or cross-sensitization is favoured when an organism is initially exposed to a high drug dose or stimulus intensity (Robinson, 1988). Accordingly, it was anticipated that the initial challenge dose of CCK-8S and the durations of stressor exposure chosen in the present Experiments (based on previous data from Experiments 1 and 3) would favour the development of cross-sensitization.

The data of Experiment 4a do not support this expectancy, however. Although animals initially challenged with 50 ng CCK-8S exhibited anxious behaviour similar to that seen in Experiment 3c, subsequent behavioural response profiles following uncontrollable footshock or restraint exposure were not influenced by prior administration of 50 ng CCK-8S. A number of variables were considered in the selection of the stressor durations for challenge. For example, stressor selection was predicated on the provocation of a mild anxiogenic response in vehicle treated animals which would not produce floor effects upon
**Figure 8a**  (Upper graph) Mean (± SEM) anxiety index (Time/sec/Transition) of CD-1 mice exposed to a no stressor condition (NS), 30 minutes of restraint stress (RS), or 30 uncontrollable footshocks (FS) or on the first test day (n=22-24/group) Behavioural tests were 10 minutes in duration.

**Figure 8b**  (Lower graph) Mean (± SEM) anxiety index (Time/sec/Transition) of CD-1 mice challenged with 5 ng CCK-8S or vehicle (Drug Challenge Condition) one week following stressor exposure (no stress (NS), 30 minutes of restraint (RS) or 30 uncontrollable footshocks (FS)). Mice were tested in the light-dark box 15 minutes following central drug administration (n=7-8/group)
challenge. Accordingly, 15 minutes of restraint (based on data of Experiment 1b) or two uncontrollable footshocks (based on results of Experiment 1a) were identified as appropriate challenge stimuli. Predictably, one week following vehicle or CCK-8S administration, two uncontrollable footshocks induced a modest anxiogenic response in CD-1 mice and 15 minutes of restraint likewise produced a small, but non-significant increase in anxiety. However, the behavior of animals in the light dark paradigm within each of these stressor conditions was independent of prior vehicle or CCK-8S administration.

Several factors may be considered in accounting for the observation that behavioral cross-sensitization was not detected in Experiment 1a. For example, the 50 ng dose of CCK-8S may not have been sufficient to provoke central neurochemical alterations consistent with the induction of behavioral cross-sensitization between CCK-8S and stressor exposure. In addition, rather than a single intraventricular injection of CCK-8S, it is conceivable that multiple exposures to the 50 ng dose of CCK-8S may have been required to foster the induction of cross-sensitization. Likewise, the stressor challenges may not have been of sufficient magnitude or duration, or previous experience in the paradigm may have led to an habituation to the test environment and a reduction of the impact of subsequent stressor challenge. As mentioned previously (Experiment 3), it should also be considered that the interval between successive challenges may have been inadequate for the expression of cross-sensitization, given the present stimulus parameters employed. Finally, it can not be discounted that, in the CD-1 mouse, perhaps cross-
sensitization between intraventricular CCK-8S administration and stressor exposure can not be demonstrated in the light dark paradigm.

In contrast to the data of Experiment 4a, the results of Experiment 4b are quite intriguing in the demonstration that initial stressor challenge may favour the development of cross-sensitization between stress and CCK-8S administration. As anticipated, the initial stressor challenges, 30 minutes of restraint or 30 uncontrollable footshocks, provoked anxiogenic behaviour in CD-1 mice in the light dark box, relative to animals in the no stress condition. However, challenge with either vehicle or 5 ng CCK-8S, one week later, produced an interesting profile. Mice injected intracerebrally with vehicle appeared unaffected, or ‘non-anxious’ and could not be differentiated on the basis of previous stressor exposure, while behaviour of some of the animals challenged with 5 ng CCK-8S did, in fact, appear to be influenced by prior stressor experience. Exposure to uncontrollable footshock or to no stress and a subsequent challenge injection of 5 ng CCK-8S resulted in behaviour comparable to that of vehicle treated animals, but mice restrained for thirty minutes one week prior to CCK-8S challenge demonstrated significant anxiogenic reactivity following 5 ng CCK-8S administration.

It is striking that the 5 ng dose of CCK-8S induced anxiety following challenge in Experiment 4b when the identical dose of CCK-8S in Experiment 3a was ineffective in producing anxiety in the light dark box on either the initial or challenge test days. Data suggest that stimuli which activate central DA systems may produce long-term alterations of neurochemical activity and hyperresponsivity to weaker stimuli presented later in time (Kalivas & Stewart, 1991, Robinson, 1988). It might be considered that the initial 30
minute restraint session may have activated the DA system in such a manner as to render the system more sensitive to subsequent CCK challenge with 5 mg CCK-8S. It will be recalled that optimal behavioural changes in anxiety were observed immediately following 30 minutes of restraint in Experiment 1b and these behavioural alterations appear to correlate with maximal increases in DA metabolites typically reported in the frontal cortex and nucleus accumbens 30 minutes after the onset of restraint (Puglisi-Allegra et al., 1991, Roth et al., 1988). These data, together with the observation that the administration of CCK and CCK agonists is also associated with increased activity of the mesocorticolimbic DA system (Crawley, 1991, 1994), support the contention that DA alterations may be involved in the anxiogenic behavioural response following restraint and subsequent CCK exposure. Although there is considerable evidence to suggest that alterations in DA neural transmission contribute to drug and stressor induced behavioural sensitization (Kalivas, Duffy, Abhold & Dilts, 1988, Kalivas & Stewart, 1991), it should also be noted that pharmacological and non-pharmacological sensitization has been detected employing indices of NE, 5-HT and GABA turnover (see Antleman, 1988 for review). In addition, the modulatory role of neuropeptides (e.g., CCK, corticotrophin releasing factor, met- and leu-enkephalin) on central activity, sensitization processes and behaviour should not be discounted (Zacharko et al., 1995). Indeed, it is likely that sensitization and cross-sensitization consists of an interactive interface between numerous transmitter and putative transmitter systems, in addition to DA. Parenthetically, it might be noted that, in some instances, cross-sensitization after drug administration and exposure to aversive environmental stimuli may be a consequence of the "stressful" nature of both treatments.
(Antleman & Caggiula, 1990) In effect, pharmacological manipulation may be interpreted as a stressor and cross-sensitization may be more likely to appear if drug administration induces neurochemical alterations similar to those associated with stressor exposure.

The observation that challenge with 5 ng CCK-8S exacerbated anxiogenic behaviour following 30 minutes of restraint, but not one week following 30 uncontrollable footshocks is also interesting. The data were not entirely unexpected, however, in view of the observation that the two stressors are fundamentally different from one another (i.e., nature of the stressor type i.e., DA turnover (Puglisi-Allegra et al., 1991)) and exposure to variable durations of footshock and restraint produced different anxiety profiles in the light dark paradigm (Experiment 1). It is conceivable that the underlying pattern of central neurochemical alterations among animals following exposure to 30 uncontrollable footshocks may have been inherently different from that of animals exposed to 30 minutes of restraint stress. Accordingly, exposure to the two stressor manipulations resulted in divergent behavioral profiles upon subsequent pharmacological challenge with 5 ng CCK-8S. The precise nature of the underlying neurochemical variations associated with the stressors employed in this Experiment and the extent to which these central alterations influence the development of cross-sensitization between stress and CCK-8S remain to be elucidated.

Although it is clear that organismic (i.e., genetics, age, sex) and environmental variables contribute to individual differences in the susceptibility to sensitization, there is little known about how these factors interact to render some individuals more sensitive to activating stimuli than others (Robinson, 1988). However, it is clear that changes in
neurotransmitter neuropeptide activity that underlie expression of behaviour can be sensitized and such neurochemical alterations may conceivably alter sensitivity to stressors, ability to cope with future stressors, and vulnerability to stressor-related disorders (Anisman & Zacharko, 1990; Antleman & Caggiula, 1990; Cassens et al., 1980). Accordingly, it should be considered that sensitization and cross-sensitization may contribute to the development and/or exacerbation of some clinically relevant syndromes, including anxiety disorders (Antleman, 1988; Kalivas & Stewart, 1991; Zacharko et al., 1995).

In summary, it has been demonstrated that stressor exposure (uncontrollable footshock and restraint) and the intraventricular administration of CCK-8S are associated with anxiogenic reactivity in CD-1 mice assessed in the light dark box model of anxiety. In an investigation of the development of cross-sensitization between stressor and CCK-8S exposure, animals challenged with either uncontrollable footshock or restraint, one week following the administration of 50 ng CCK-8S, did not demonstrate exacerbated anxiogenic reactivity to the stressor challenge. However, when challenged with a low dose of CCK-8S subsequent to a specific stressor manipulation, behavioural cross-sensitization was detected. Specifically, mice previously restrained for 30 minutes exhibited significant anxiety following challenge with 5 ng CCK-8S, a dose which has been shown to be ineffective at promoting behavioural alterations, while the behaviour of previously shocked animals did not differ from that of non-stressed animals following CCK challenge.
GENERAL DISCUSSION

The data of the present series of Experiments provide initial evidence demonstrating the anxiogenic effects of nanogram doses of CCK-8S, administered intraventricularly in mice in the light dark paradigm. Behaviour was not affected by peripheral receptor activation, which is invariably induced following systemic drug administration, since central CCK$_{11}$ receptors were activated directly. In addition, owing to the nanogram doses of CCK-8S selected, transfer of the peptide from cerebrospinal fluid to the periphery can not be readily cited in accounting for behavioural change as might be expected when larger doses of the peptide are administered centrally.

Animals were invariably anxious following the administration of the highest dose of CCK-8S employed (50 ng), while behaviour remained unaltered after the administration of the lowest dose (5 ng). However, it is noteworthy that the 25 ng challenge dose of CCK-8S augmented anxiety in the light dark paradigm, when the identical dose did not provoke an anxiogenic response on the initial test day, one week previously. In essence, the 25 ng challenge dose of CCK-8S was sufficient to increase anxiety in mice if these animals were either exposed to the light dark paradigm previously or treated with 25 ng CCK-8S of the anxiogenic one week earlier. The saliency of these variables do not appear trivial, considering an anxiogenic effect was not elicited with other doses of CCK-8S and previous exposure to the light dark box. Although, these data may be specific to the CD-1 mouse in this particular paradigm, the 25 ng dose of CCK-8S appears to approximate a pharmacological threshold for central CCK$_{11}$ receptor activation and the provocation of anxiogenic behaviour.
In addition to the evaluation of the behavioural effects of nanogram doses of CCK-8S, an investigation of behavioural cross-sensitization between stressor and CCK-8S exposure was also conducted. Remarkably, 5 ng CCK-8S, the dose which was shown to be ineffective at promoting behavioural alterations, provoked anxiety in animals which had previously been restrained. However, increased anxiety following 5 ng CCK-8S challenge was only demonstrated if animals had been restrained one week previously, not if they had been exposed to uncontrollable footshock. It should be considered that the nature of the particular stressor employed, rather than stressor presentation per se, appears fundamental to the expression of subsequent behavioural reactivity. In retrospect, it is conceivable that restraint may be more relevant, or significant to a rodent than uncontrollable footshock. In effect, since cross-sensitization may serve a protective function from an evolutionary perspective, the development of sensitization may be favoured by exposure to stimuli which are ethologically more meaningful or threatening to the species.

Although cross-sensitization was demonstrated between restraint and subsequent central CCK-8S administration, augmented anxiogenic behaviour was not in evidence when CCK-8S was administered initially and animals were challenged with a stressor one week later. It is interesting that behavioural cross-sensitization was established in only one direction. These results could be paradigm or species specific, or perhaps cross-sensitization between CCK-8S and subsequent stressor exposure might be demonstrated in the light-dark paradigm under different experimental conditions. However, these data underscore the importance of the precise stimulus parameters employed and the sequence of stimulus presentation to the detection of anxiogenic behaviour.
It might be considered that if variant forms of cross-sensitization can be demonstrated, these data may have implications for the human condition. It is conceivable that at least two neurochemically relevant types of anxiety may be expressed - one of these may pertain to individuals vulnerable to the effects of aversive life events (i.e., paralleled by the stressor - CCK-8S condition), while the other may include individuals with an endogenous neurochemical imbalance or vulnerability (i.e., similar to the CCK-8S - stressor condition). In fact, it has been suggested that pathological forms of anxiety might stem from a hyperactivity of the CCK$_R$ system or an imbalance between central CCK$_A$ and CCK$_R$ receptor activity (Harro et al., 1993). Accordingly, varying predisposing etiologies and distinct behavioural symptoms would suggest variable therapeutic strategies among individuals of such populations (e.g., anxiolytics, antidepressants or CCK$_R$ antagonists) if, in fact, both forms of anxiety could be treated through pharmacological interventions.

It should also be noted parenthetically, that repeated exposure to aversive stimuli may not only increase the magnitude and decrease the latency of behavioural symptoms through sensitization, but may also enable cues associated with stressors to provoke behavioural and/or neurochemical alterations associated with sensitizing stimuli (Post & Weiss, 1989). Consequently, seemingly innocuous events or stimuli may also come to induce behavioural change. In this respect, it would be relevant to consider that, in humans, anxiogenesis may be provoked and/or exacerbated by exposure to a multitude of cues or stimuli which may precipitate behavioural symptoms through conditioning and sensitization.
In summary, the present series of Experiments has identified the threshold dose of an anxiogenic agent and the specific stimulus parameters which favor the induction of cross-sensitization between stressors and an anxiogenic agent in the CD-1 mouse in the light dark paradigm. Extrapolating to the human condition, it might be considered that anxiety may only become evident when a particular set of conditions have been met (i.e., when a threshold has been exceeded or specific stimuli have been encountered) and that these conditions may differ for each individual. Of course, age, sex, genetic predisposition, coping strategies and prior stressor history, among other variables, interact in rendering some individuals more sensitive to activating stimuli than others. The challenge lies in unraveling the underlying causes and predisposing factors, identifying the particular conditions and parameters associated with the exacerbation of anxiety-related disorders and determining an appropriate treatment regimen for each affected individual.
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Appendix A - ANOVA Tables
### Experiment 1A - ANOVA Tables for Baseline Anxiety Analysis

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**Experiment 1A - ANOVA Tables for Post Shock Anxiety Analysis**

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Experiment 1B - ANOVA Tables for Post Restraint Anxiety Analysis

**Between Group Effects**

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**Within Group Effects**

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### Experiment 2 - ANOVA Tables for Post Shock Anxiety Analysis

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### Experiment 3A - ANOVA Tables for Post CCK (5 ng) Anxiety Analysis

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#### Within Group Effects

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### Experiment 3B - ANOVA Tables for Post CCK (25 ng) Anxiety Analysis

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**Experiment 3C - ANOVA Tables for Post CCK (50 ng) Anxiety Analysis**

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**Within Group Effects**

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<td>31</td>
<td>0.0347</td>
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<td>MS=79.983240</td>
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<tr>
<td>D. x Init. x Chall.</td>
<td>SS=32.802153</td>
<td>0.77</td>
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<tr>
<td></td>
<td>MS=16.401076</td>
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<td>Error</td>
<td>SS=660.84796507</td>
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<tr>
<td></td>
<td>MS=21.31767629</td>
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### Experiment 4A - ANOVA Tables for Post CCK-Stress Anxiety Analysis

#### Between Group Effects

<table>
<thead>
<tr>
<th>Effect</th>
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<th>F</th>
<th>DF</th>
<th>DF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grand Mean</td>
<td>SS=4513 137427, MS=4513 137427</td>
<td>221.69</td>
<td>1</td>
<td>38</td>
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<tr>
<td>Drug</td>
<td>SS=52 242514, MS=52 242514</td>
<td>2.57</td>
<td>1</td>
<td>38</td>
<td>0.1174</td>
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<tr>
<td>Stress</td>
<td>SS=64 416928, MS=32 208464</td>
<td>1.58</td>
<td>2</td>
<td>38</td>
<td>0.2188</td>
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<tr>
<td>Drug x Stress</td>
<td>SS=5 193752, MS=2.596876</td>
<td>0.13</td>
<td>2</td>
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<td>0.8806</td>
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<td>SS=773 58928726, MS=20 35761282</td>
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#### Within Group Effects

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Day</td>
<td>SS=11.404846, MS=11.404846</td>
<td>0.89</td>
<td>1</td>
<td>38</td>
<td>0.3507</td>
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<tr>
<td>Day x Drug</td>
<td>SS=135 136523, MS=135 136523</td>
<td>10.58</td>
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<tr>
<td>Day x Stress</td>
<td>SS=96 947222, MS=48 473611</td>
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<tr>
<td>D x Drug x Stress</td>
<td>SS=0.199476, MS=0.099738</td>
<td>0.01</td>
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<tr>
<td>Error</td>
<td>SS=485 37787452, MS=12 77310196</td>
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**Experiment 4B - ANOVA Tables for Post Stress-CCK Anxiety Analysis**

### Between Group Effects

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<tbody>
<tr>
<td>Grand Mean</td>
<td><strong>SS=4440 302931</strong>&lt;br&gt;<strong>MS=4440 302931</strong>&lt;br&gt;333 04</td>
<td>1</td>
<td>40</td>
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<tr>
<td>Stress</td>
<td><strong>SS=142 361430</strong>&lt;br&gt;<strong>MS=71 180718</strong>&lt;br&gt;5 34</td>
<td>2</td>
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<tr>
<td>Drug</td>
<td><strong>SS=37 800031</strong>&lt;br&gt;<strong>MS=37 800031</strong>&lt;br&gt;2 84</td>
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<td>40</td>
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<tr>
<td>Stress x Drug</td>
<td><strong>SS=45 490334</strong>&lt;br&gt;<strong>MS=22 745167</strong>&lt;br&gt;1 71</td>
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<td>Error</td>
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### Within Group Effects

<table>
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<tbody>
<tr>
<td>Day</td>
<td><strong>SS=73 344452</strong>&lt;br&gt;<strong>MS=73.344452</strong>&lt;br&gt;14 93</td>
<td>1</td>
<td>40</td>
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<tr>
<td>Day x Stress</td>
<td><strong>SS=43 524727</strong>&lt;br&gt;<strong>MS=21.762363</strong>&lt;br&gt;4 43</td>
<td>2</td>
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<td>Day x Drug</td>
<td><strong>SS=63 772800</strong>&lt;br&gt;<strong>MS=63.772800</strong>&lt;br&gt;12 98</td>
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<td>D x Stress x Drug</td>
<td><strong>SS=41 305090</strong>&lt;br&gt;<strong>MS=20.652545</strong>&lt;br&gt;4 20</td>
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<td>Error</td>
<td><strong>SS=196.52196103</strong>&lt;br&gt;<strong>MS=4.91304903</strong>&lt;br&gt;</td>
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