Effects of Cholecystokinin and Cholecystokinin Antagonists on Isolation-Induced Ultrasonic Vocalizations in Rat Pups

by

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ABSTRACT

Rat pups emit ultrasonic vocalizations (UVs) when separated from the dam and littermates. Some investigators have suggested that isolation-induced UVs signify a behavioural manifestation of distress or anxiety. Many compounds that possess anxiolytic properties reduce isolation-induced UVs, whereas anxiogenic drugs increase UVs. The CCK tetrapeptide fragment (CCK-4) induces panic in humans, and individuals diagnosed with panic disorder exhibit a heightened sensitivity to the anxiogenic effects of CCK-4. However, the effects of CCK in traditional animal models of anxiety have been variable, and data examining the effects of CCK on isolation-induced UVs is limited. Previous studies have demonstrated an anxioselective effect for CCK-8s in 11-day-old rats (Weller & Blass, 1988), although negative results have been reported with younger pups (Rex, Barth, Voight, Domeney, & Fink, 1994; Weller & Dubson, 1998). CCK-4 has been shown to increase UV rates (Rex et al., 1994) in 5-day-old rats. The current series of experiments was designed to assess the effects of various CCK fragments and antagonists on isolation-induced UVs, motor activity, and body temperature in 12-day-old, Long Evans hooded rats. It was hypothesized that CCK-4 would increase, whereas CCK-8s would decrease, UV rates. In Experiments 1-4, subjects were treated with BOC-CCK-4 (which acts on CCK-2 receptors) or CCK-8s (which acts on CCK-1 and CCK-2 receptors), and UVs, motor activity, and body temperature were examined 15 or 30 minutes following drug pre-treatment. Contrary to expectations, BOC-CCK-4 failed to exert any significant effects on UVs (Experiments 1 and 2), and CCK-8s, administered 15 minutes prior to testing, was also without effect on call rates (Experiment 3). In Experiment 4, CCK-8s (30 minutes post-treatment) reduced UV rates at each of the doses tested (0.5, 1.0, 2.0 and 4.0 μg/kg). This effect on UVs was specific in that motor activity and body temperature were unaffected. In Experiments 5 and 6, pups were pre-treated with vehicle, devazepide (a CCK-1 receptor antagonist), or L-365,260 (a CCK-2 receptor antagonist), respectively, 45 minutes prior to testing. Pups in both experiments were subsequently treated with vehicle or CCK-8s (2.0 μg/kg), 30 minutes prior to testing. It was hypothesized that one or both of the antagonists would block the UV effects associated with CCK-8s treatment. However, neither devazepide nor L-365-260 exerted any significant effects on UVs, and the reduction in UVs following CCK-8s pre-treatment observed in Experiment 4, was not replicated. In Experiment 5, subjects treated with devazepide (1000 μg/kg)/CCK-8s engaged in significantly less wall climbing compared to devazepide/vehicle-treated subjects. In Experiment 6, pups that were administered L-365,260 (1000 μg/kg)/CCK-8s exhibited fewer line crossings compared to pups treated with L-365,260/vehicle. Body temperature levels were not affected in Experiment 5 and 6. Under the parameters of the current set of experiments, the isolation-induced UV model possesses limited utility for studying the potential contribution of CCK in modulating anxiety-related behaviour.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ACR	Abdominal Compression Reaction
α-2	Alpha-2
ANOVA	Analysis of Variance
BAT	Brown Adipose Tissue
β-CCE	Methyl-β-carboline-3-carboxylate
β-1	Beta-1
BOC-CCK-4	Butyloxycarbonyl-Cholecystokinin-4 (octapeptide)
°C	Degrees Celsius
cm	Centimetres
CCK	Cholecystokinin
CCK-A	Cholecystokinin-A
CCK-B	Cholecystokinin-B
CCK-4	Cholecystokinin-4 (tetrapeptide)
CCK-8s	Cholecystokinin-8 (octapeptide) sulphated
CCK-8us	Cholecystokinin-8 (octapeptide) unsulphated
CCK-1	Cholecystokinin-1
CCK-2	Cholecystokinin-2
CRF	Corticotrophin Releasing Factor
DA	Dopamine
Db	Decibel

DMSO	Dimethylsulfoxide
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
EAA	Excitatory Amino Acid
5-HT	Serotonin
5-HT_{1A}	Serotonin 1A
5-HT_{2A}	Serotonin 2A
GABA	Gamma-Aminobutyric Acid
i.p.	Intraperitoneally
KHz	Kilohertz
L-365,260	3R-3-[N'-(3-methylphenyl) ureido]-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one
mRNA	Messenger Ribonucleic Acid
µg/kg	Microgram per kilogram
µl	Microlitre
mg/kg	Milligram per kilogram
ml/kg	Millilitre per kilogram
ms	Milliseconds
MDTB	Mouse Defensive Test Battery
NE	Norepinephrine
NST	Non-Shivering Thermogenesis
OLETF	Otsuka Long Evans Tokushima Fatty

PAG	Periaqueductal Gray
PLSD	Post-Hoc Least Significant Difference
SSRIs	Selective Serotonin Re-uptake Inhibitors
UV	Ultrasonic Vocalization
UVs	Ultrasonic Vocalizations
γ	Gamma
$ZnSO_4$	Zinc sulphide

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1.0. GENERAL INTRODUCTION

1.1 Isolation-Induced Ultrasonic Vocalizations (UVs) in Rat Pups

1.1.1. General Background and Developmental Considerations

Pups from numerous rodent species produce ultrasonic vocalizations (UVs), which are high frequency calls that fall beyond the audible range of humans (i.e., above 20 kHz). The quantitative and qualitative characteristics of UVs have been documented in mice and voles (Zippelius & Schleidt, 1956), gerbils (Sewell, 1968), shrews and hamsters (Kahmann & Ostermann, 1951), and rats (Noirot, 1968), and comparative reviews have been carried out that have examined UVs across different rodent species (e.g., Anderson, 1954; Motomura et al., 1972; Sales & Smith, 1978; Sewell, 1968).

The current research will focus on UVs emitted by rat pups. Numerous anatomical sites have been implicated to play a role in the production of UVs in rats (see Hofer, 1996 for a review). In this respect, the larynx is crucial. Roberts (1972, 1975a,b,c) demonstrated that UVs result from air passing through two constricted laryngeal plates during the initial phases of expiration. Wetzel, Kelley, and Campbell (1980) reported that in 10-day-old rat pups, unilateral or bilateral transection of either the inferior or superior aspects of the laryngeal nerve exerted significant effects on UV production. Specifically, transection of the inferior laryngeal nerve resulted in a complete cessation of UV production (although pups still emitted audible squeaks). Transection of the superior

laryngeal nerve reduced the sound pressure level and rate of UV production, and increased the fundamental frequency of the calls (again, pups remained capable of emitting audible squeaks). By examining patterns of retrograde transport of horseradish peroxidase, Wetzel et al. (1980) demonstrated that the inferior laryngeal nerve receives projections from the dorsal formation of the nucleus ambiguous of the brainstem, whereas the superior laryngeal nerve receives projections of from the ventral aspect of the nucleus ambiguous.

The nucleus ambiguous receives projections from the reticular formation, which in turn receives input from the midbrain periaqueductal grey (PAG) area (Hofer, 1996). The PAG represents an integration point for both ascending sensory input (from both the external environment and the viscera) via spinal accessory pathways and descending input from a number of brain regions (i.e., the anterior cingulate cortex, nucleus accumbens, preoptic area, amygdala, septum, thalamus, hypothalamus, and the superior and inferior colliculi), many of which are involved in the mediation of motivational and affective behaviour (see Hofer, 1996 for a review of the neuroanatomical loci involved in UV production). Consistent with the finding that the PAG integrates such diverse central and peripheral efferents, specific longitudinal segments of this region have been implicated in the mediation of different behaviours including analgesia, autonomic regulation, lordosis, vocalizations, and fear- and anxiety-related behaviour (Bandler & Shipley, 1994; Behbehani, 1995; Depaulis, Keay, & Bandler, 1992; Jürgens, 1994; Yajima, Hayashi, & Yoshii, 1980). Wiedenmayer,

Goodwin, and Barr (2000) reported a direct link between the PAG and UVs in rat pups by demonstrating that electrolytic lesions of either the lateral or ventrolateral PAG resulted in reduced rates of calling in 10-day-old Long Evans hooded rat pups. Taken together, these data indicate that the neural control of UV production stems from the co-ordinated involvement of many central and peripheral loci (see Figure 1).

It is not surprising to find that the neuropharmacological basis of UV production is also complex, and that exogenous pharmacological compounds acting on many different neurochemical systems exert differential effects on isolation-induced UVs. These data will be considered in a subsequent section (see section I. D.).

In rat pups, the emission of UV is influenced by a number of factors, including tactile stimulation (Okon, 1972; Oswalt & Meier, 1975; Sales & Smith, 1979), olfactory cues (Conely & Bell, 1978; Oswalt & Meier, 1975; Richardson & Defina, 1998; Takahashi, 1994), footshock stress (Takahashi, Turner, & Kalin, 1991), and maternal nutritional variables (Hunt, Smotherman, Weiner, & Levine, 1976). Separation from the dam and littermates (Allin & Banks, 1971; Okon, 1971; Sales & Smith, 1978) results in the production of "isolation-induced UVs", which will be the focus of the current line of research. The quantitative and qualitative characteristics of isolation-induced UVs in rat pups have been shown to vary between different species of rats (Watts, 1980), between strains within the same species (Nitschke, Bell, Bell, & Zachman, 1975; Sales, 1979), between

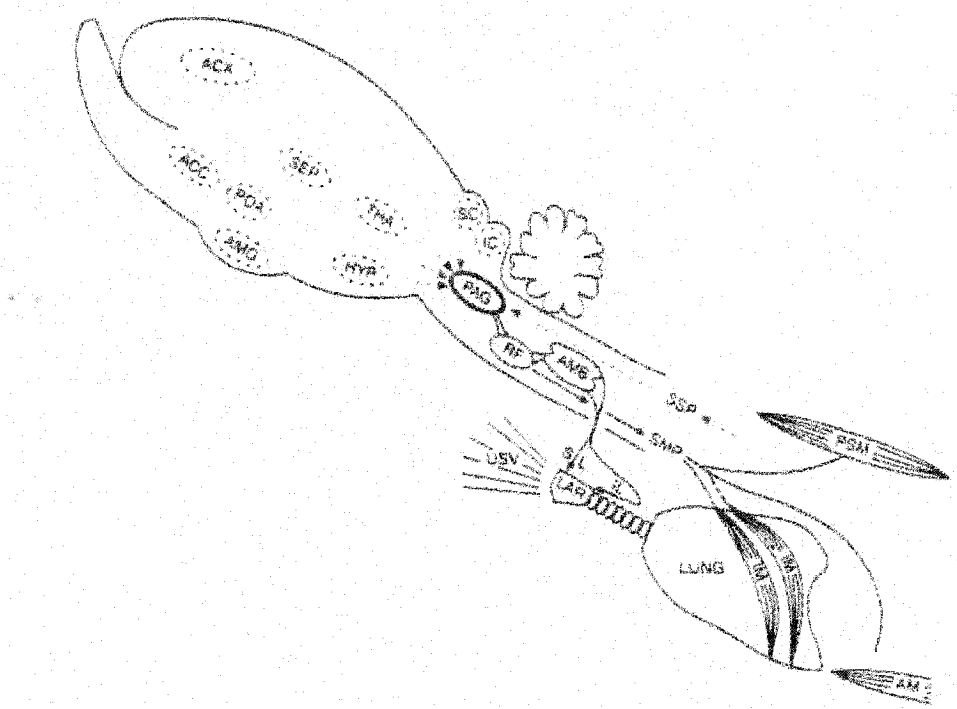


Figure 1*: A schematic model for the neuroanatomical structures and pathways mediating mammalian vocalization. The output system, originating in the periaqueductal grey (PAG), is depicted in solid lines. Symbols are as follows: ACX, anterior cingulate cortex; PAG, periaqueductal grey; ACC, nucleus accumbens; RF, reticular formation; SEP, septal area, AMB, nucleus ambiguous. POA, preoptic area; SL, superior laryngeal nerve; AMG, amygdala; IL, inferior laryngeal nerve, THA, dorsal thalamus; LAR, larynx; HYP, hypothalamus; SMP, spinal motor pathways; SC, superior colliculus; SSP, spinal sensory pathways; IC, inferior colliculus; PSM, paraspinal muscles; IM, intercostal muscles; AM, abdominal muscles.

***Modified from Hofer (1996)**

litters from the same strain, and even within litters (Brunelli, Vincoor, Soo-Hoo, & Hofer, 1997; Graham & Letz, 1979). Despite this variability, some characteristic features have emerged. Isolation-induced UV rates are initially low after birth, but then increase significantly with the unfolding of the external ears (corresponding to postnatal days 2-3). Rates of calling then gradually increase over subsequent days, peaking from days 4-9. Then a gradual reduction in calling typically takes place, with a significant decrease after eye opening occurs (i.e., days 14-17). By day 21, UV production essentially stops altogether (Noirot, 1968).

Rat pup UVs are typically brief, and emitted in pulses at approximately 60-80 dB (Branchi, Santucci, & Alleva, 2001; Hofer, 1996; Hofer & Shair, 1978). The majority of rat pup UVs occur within the 40-50 kHz frequency range. However, as pups age, the kHz frequency of calls among pups becomes increasingly variable (Noirot, 1968). Consistent with this finding of increased frequency variability with advancing age, Brudzynski, Kehoe, and Callahan (1999) reported age-related increases with respect to the difference between the lowest and highest frequencies amongst 10-, 15-, and 17-day-old Sprague Dawley rat pups. Analysis of the associated call sonograms revealed that as pups age, they tend to produce sweeping calls, which carried across sound frequencies (i.e., represented on the sonogram as a "U" or an "inverted U"). In addition, age-associated changes have also been detected with respect to the peak call frequency (increasing from 50 kHz to 64 kHz), the mean duration of calls

(increasing from 80 ms to 140 ms) (Brudzynski et al., 1999), and the duration of the inter-call interval (Elsner, Suter, & Alder, 1990). Conversely, the mean call frequency has been reported to decrease with age (Elsner et al., 1990). Brudzynski and colleagues hypothesised that such age-related changes in the qualitative aspects of UVs represent a refinement of the most important (from a survival perspective) acoustic features of isolation-induced UVs. Of note, in the Brudzynski et al. study, data obtained from pups in the 10-day-old group were further analysed for inter-litter differences. Consistent with previous findings documenting significant inter-litter differences in call rates, pups from separate litters also differed with respect to call duration, bandwidth, and peak frequency. The authors suggested that such inter-litter differences might serve to maximise the likelihood that dams will recognise UVs made by pups from their own litter (Brudzynski et al.).

1.1.2. The Influence of UVs on Maternal Behaviour

UVs exert significant facilitative effects on different aspects of maternal behaviour, which in turn, play a significant role in the neurobehavioral development of the pup (Branchi et al., 2001; DeGhett, 1978; Hofer, 1996 Noirot, 1972; Bell, 1979; Sales & Smith 1978). As a result, UVs have been recognised as an important adaptive behaviour during early development in the rat (see Hofer 1994, 1996). Noirot (1972) categorised the behavioural effects of UVs on adult (usually maternal) behaviour as either "withdrawal" or "positive" effects. Withdrawal effects are those behaviours that involve a cessation of a

behaviour on the part of the adult, for example rough handling (sitting or stepping on the pup). In this respect, when Long Evans hooded dams were prevented from hearing pups' broadband vocalizations, (which included both audible and ultrasonic components), following either the implantation of ear plugs in the dam (Inhat, White, & Barfield, 1995) or after the pup had been anesthetized or had its mouth sealed with an adhesive substance (White, Adox, Reddy, & Barfield, 1992), the dams sat on or stepped on the pups more often. UV emission was hypothesised to reduce such contact, thus reducing the likelihood of accidental injury to the pup. In contrast to withdrawal effects, positive effects, according to Noirot (1972), consist of instances where the adult becomes attracted towards the pup after being exposed to UVs (e.g., resulting in search and retrieval behaviour and/or nest building).

When considering the effects of isolation-induced UVs on maternal behaviour, the resultant maternal behaviours would invariably fall in the category of positive effects, given that pups are physically separated from the dam (i.e., and thus withdrawal, or cessation of an ongoing behaviour is not possible). Once a pup is retrieved by the dam, it emits fewer UVs, so while isolation-induced UVs affect maternal behaviour, the subsequent response of the dam then influences UV production. While experiential factors have been shown to influence UV rates (see Hofer 1996 for a review), isolation-induced UVs are also produced by Wistar pups that have been artificially reared (i.e., without contact with the dam or littermates), even from as early as the day the pups were born (Hofer, Brunelli,

& Shair, 1993a; Hofer, Shair, & Murowchick, 1989). These findings suggest that UV production following isolation is, at least in part, genetically programmed.

Allin and Banks (1972) reported that lactating Wistar rat dams oriented their bodies more frequently to the sound of recordings of isolation-induced UVs produced by a 7-day-old pup, compared to a control recording (background noise). This differential behavioural response was not evident amongst adult male or virgin female rats. In addition, lactating dams exposed to the UV recording tended to be more accurate in orienting toward the source of the recorded UVs. Finally, the dams exhibited a tendency to leave the nest box and engage in searching behaviour, while males and virgin females did not. However, dams did not consistently engage in searching behaviour when exposed to the recorded UVs, and the authors hypothesised that this finding may have been due to a lack of associated pup olfactory cues that are typically available to the dam (Allin & Banks, 1972).

In keeping with a hypothesized role for olfactory cues in mediating the maternal response to pup UVs, Smotherman, Bell, Starzec, Elias, and Zachman (1974) found that olfactory cues from Long Evans rat pups were necessary in order to provoke lactating dams to engage in pup searching behaviour in a Y-maze paradigm. However, the additional presence of UVs (either recorded from isolated pups, or produced by actual pups that were being handled by the experimenter) further increased the likelihood that dams would search for the pups, and were judged to serve as "effective directional cues to elicit retrieval"

(pg. 61). Brunelli, Shair, and Hofer (1994) reported that Wistar rat dams responded more frequently in a Y-maze paradigm (i.e., more maze arm entries and longer time spent in the arm) to cooled, vocalising pups, compared to cooled, anesthetized (i.e., non-vocalising) pups. Given that olfactory cues were present in both arms of the maze, these findings provide further evidence that pup UVs influence maternal behaviour. Subsequent studies have demonstrated that UVs prompt the Wistar rat dams to return to the nest following an absence (Jans & Leon, 1983), facilitate the retrieval and transport of pups that are concealed within the nest (Brewster & Leon, 1980), and induce maternal anogenital licking of pups. Licking of Wistar rat pups by the dam has been demonstrated to stimulate pup urination and defecation (Brouette-Lahlou, Vernet-Maury, & Vigouroux, 1992), and to affect gene expression underlying neurochemical and behavioural responsivity to stress in the pup (see Meaney, 2001 for a review). These findings further highlight the reciprocal nature of the UV-mediated relationship between the pup and dam. UVs modify the behaviour of dams, and such behavioural changes on the part of the dam (e.g., increased licking of the pup) result in significant changes to the overall development of the pup.

Isolation-induced UVs affect a neuroendocrine response in lactating dams. Terkel, Damassa, and Sawyer (1979) reported that lactating Sprague-Dawley rat dams reacted with elevated plasma prolactin levels following exposure to a taped, 15-minute recording of isolation-induced UVs produced by 7-day-old pups

that were placed on a cold plate. In contrast, elevated plasma prolactin levels were not recorded following maternal exposure to either of the control conditions (i.e., taped, adult UVs, or taped background noise). Of note, virgin females also showed a slight, yet statistically significant increase in plasma prolactin levels following exposure to the taped UVs. Based on these findings, the authors hypothesised that the prolactin response to UVs "...may represent an inherent characteristic which is enhanced after parturition" (Terkel et al., 1979, p. 100).

These effects of UVs on prolactin levels were not replicated by separate groups of investigators. Voloschin and Tramezzani (1984) failed to detect a rise in the prolactin levels of lactating Holtzman rats following exposure to pup UVs associated with returning the pups to the litter. However, these authors noted that the eliciting stimuli for pup UVs differed in their study compared to Terkel et al. (1979), which may have accounted for the discrepant findings. Stern, Thomas, Rabii, & Barfield (1984) tested lactating Sprague Dawley rat dams at 1 or 2 weeks postpartum, and also failed to detect a significant increase in dam's prolactin levels in response to UVs recorded from cooled 7-day-old pups. The lack of replication regarding UV-induced prolactin release in this study calls into question Terkel et al.'s view that such a neuroendocrine response is an "inherent characteristic" amongst female rats. However, significant procedural differences between the studies were noted, including the fact that Terkel and colleagues tested dams repeatedly across test days and treatment conditions. In contrast, in the Stern et al. (1984) study dams were assessed on only one day following

exposure to a single treatment condition. This discrepancy raises the possibility that the elevated prolactin response amongst dams reported by Terkel et al. may have been due, at least in part, to physiological sensitisation resulting from the repeated exposure to distressing pup calls. However, Hashimoto, Saito, Furudate, & Takahashi (2001) reported significant elevations in plasma prolactin concentrations from Wistar-Imamichi rat dams, accompanied by simultaneous increases in aspects of maternal behaviour (i.e., search and retrieval behaviour and nest building), following exposure to the UVs emitted by pups that were isolated in a beaker placed on an ice bag. In this study, dams were tested only once, thus eliminating the possibility that the observed endocrinological (and behavioural) responses to UVs may have resulted from physiological sensitisation. For the purposes of the current research, the nature of the prolactin response to UVs (i.e., an inherent response, learned response, or combined inherent/learned response) is secondary. Of primary importance is the fact that UVs provoke physiological and behavioural changes in the dam that are vital to pup survival.

1.1.3. Conflicting Views Regarding the Causal Mechanisms Underlying Isolation Induced UVs

As previously noted, numerous factors (i.e., thermal, tactile, olfactory cues) appear to be involved in the elicitation of isolation-induced UVs. These calls influence maternal behaviour and physiology, which in turn have significant adaptive consequences for the pup. Some researchers have hypothesised that

isolation-induced UVs represent the behavioural manifestation of distress or anxiety, arising from the removal of familiar sensory cues from the pup's environment, and disruption of the attachment bond between the pup and the dam (e.g., Hofer 1994, 1996; Okon, 1972). Once this signal is received by the dam, she responds in such a way as to relieve the pup's level of distress (i.e., by retrieving the pup and returning it to the thermal, tactile, and olfactory cues associated with the nest). This interpretation stresses that isolation-induced UVs are primarily a form of social communication, and would suggest that the study of UVs might represent a valid method by which to examine issues related to the neurobiology of anxiety.

In contrast to this view, it has been suggested that UVs are merely an acoustic by-product, not unlike a cough or sneeze, resulting from physiological changes that occur in the rat pup, in response to decreased body temperature associated with isolation. According to proponents of this view, focusing on underlying physiological processes (rather than presumed emotional states) eschews the need to adopt an anthropomorphic approach when trying to determine what causes isolated pups to emit UVs (Blumberg, 1992; Blumberg & Alberts, 1990, 1991a, 1997; Blumberg & Sokoloff, 2001, 2003; Blumberg, Sokoloff, Kirby, & Kent, 2000). While this view does not discount that UVs produced during isolation possess communicatory value (as evidenced by the effects of UVs on behaviour of the dam), it is suggested that UVs have developed their communicatory value through a process of "exaptation" (Gould 1991; Gould

& Vrba, 1982), whereby the behaviour's original function (i.e., as part of a chain of physiological events employed to respond to cold temperatures) has resulted in a serendipitous effect (i.e., retrieval by the dam). These conflicting viewpoints will now be considered in turn.

1.1.3.1. Isolation-Induced UVs as an Acoustic By-Product of Physiological Changes Resulting from Decreased Body Temperature

Early studies regarding the ontogeny of isolation-induced UVs in rat pups demonstrated a strong relationship between ambient temperature and UV production (Allin & Banks, 1971; Noirot 1972; Okon, 1971, 1972). Specifically, Allin and Banks (1971) isolated Wistar rat pups at 2°C, 20°C, 35°C, or 40°C, and recorded UVs over a 4.5-minute period. The 2°C and 20°C conditions were chosen in order to induce differential degrees of decline in body temperature. In contrast, the 35°C condition represented a thermoneutral zone, similar to the temperature in the nest in the presence of the dam and littermates (Galineo & Galineo, 1952, Taylor, 1960). Finally, the 40°C condition was chosen in order to assess the potential impact of an abnormally elevated ambient temperature on isolation-induced UVs.

At 2°C and 20°C, UV production increased from days 2-6, with peak values reached at days 6-8. This peak range is generally consistent with the peak range of days 4-9, as reported by Noirot (1968). UV rates then decreased steadily, reaching near-zero levels by day 18 (again, in general agreement with the results of Noirot [1968]). Allin and Banks (1971) suggested that the decline in calling

was possibly related to the "...diminishing threat of low ambient temperature" (p. 155), coincident with the ongoing development of homeothermic abilities in the pup.

At 35°C and 40°C, calling was infrequent until approximately 10-12 days, at which time there was an increase. Given that relatively lower rates of UVs were produced by pups in the 35°C (i.e., thermoneutral) group, it was suggested that non-thermal factors also contributed to some degree to elicit isolation-induced UVs, although this effect was generally not present until pups became older. In this respect, when discussing the possible factors associated with UV production by pups in the thermoneutral group, Allin and Banks (1971) stated "Pups may call in the absence of physical contact with the mother and littermates, because of hunger, or in response to extreme levels of relative humidity. However, if such factors are indeed operative, they are patently less effective than low ambient temperatures in evoking high call rates." (p. 155).

The relationship between ambient temperature, the development of homeothermy, and the rate of UV production was further reinforced by the work of Okon (1971, 1972). Wistar rat pups were isolated at one of three different ambient temperatures (2-3°C, 12°C, or 22°C) for one hour per day, over 20 days. Body temperature at the conclusion of the one-hour test session was recorded, and compared across days in order to determine the age at which pups in the different treatment groups began to exhibit homeothermic capabilities. During the first few postnatal days UV production was virtually absent. This finding has

been attributed to the fact that rat pups at this developmental stage are poïkilothermic; they are unable to regulate their body temperature at all, and thus rapidly enter into a coma state and stop breathing and calling (see Noirot, 1972 for review). Subjects isolated at 2-3°C did not demonstrate a significant homeothermic response until day 14, and relatively high UV rates were also evident until that time. In contrast, pups isolated at 12°C or 22°C exhibited homeothermic capabilities (accompanied by relatively lower UV rates) starting by days 6-8. Taken together, these data indicate that the effect of ambient temperature on isolation-induced UVs varies as a function of the age (and thus the homeothermic capacity) of the pup being tested (Okon, 1971, 1972). When interpreting the relationship between the development of homeothermy and isolation-induced UVs, Okon (1972) stated that "...infant rodents possess a way of *calling for help* [italics added] by the emission of ultrasonic signals" (p. 146), suggesting that UV production in response to isolation might serve as a behavioural means by which to cope with the demands of isolation.

While considering the relationship between thermoregulatory abilities and UV production, Blumberg and Alberts (1990) investigated the interaction between UV production and several physiological mechanisms known to be involved in thermoregulation. They noted that in the rat, the ability to increase body temperature is mediated primarily by non-shivering thermogenesis (NST; Taylor, 1960), produced by a specialised heat-producing organ, brown adipose tissue (BAT; Smith 1964; Smith & Roberts, 1964). BAT consists of highly

vascularised tissue, and it is located in the superior cervical, interscapular, and thoracic regions. When activated by cold, oxygen delivery to BAT increases, and thermogenesis ensues, which results in selective heating of the vasculature supplying blood to vital organs (including the heart), segments of the spinal cord, and the sympathetic chain.

Taking the above noted factors into consideration, Blumberg and Alberts (1990) demonstrated that when 10- to 12-day old Sprague-Dawley rat pups were isolated in a thermoneutral environment (i.e., 35°C), body temperature and oxygen consumption values remained steady and UVs were, for the most part, absent. However, once the air temperature was reduced, a series of physiological changes was initiated; body temperature decreased, BAT was activated, and respiration frequency and oxygen consumption increased. At the same time, the rat pups started to produce UVs. The authors noted that UVs were produced during periods of prolonged expiration, and thus suggested that UVs might result from a respiratory manoeuvre, performed in order to enhance oxygen intake and to accommodate the metabolic demands associated with thermogenesis. In this respect, the periods of prolonged expiration during which UVs were produced closely resembled a respiratory manoeuvre known as laryngeal braking. As previously noted, laryngeal involvement in UV production was clearly established by Roberts (1972, 1975a,b,c). Laryngeal braking has been associated with increased intrathoracic pressure and improved gas exchange in the lungs, which improves respiratory efficiency and oxygen delivery to tissue (Davis & Bureau,

1987; England, Kent, & Storyn, 1985; Gauthier, Remmers, & Bartlett, 1973). When considering the isolated and cold pup, laryngeal braking would thus facilitate oxygen transfer to BAT and improve a pup's ability to cope with colder temperatures (Blumberg & Alberts, 1990).

Consistent with their hypothesised relationship between the metabolic demands associated with thermoregulation and UV production, Blumberg and Alberts (1991b) demonstrated that either the induction of hypoxia or milk deprivation (both of which decrease metabolic demands) resulted in concomitant decreases in the amount of time that isolated Sprague-Dawley pups spent vocalising. In addition, allowing pups to huddle with littermates, which inhibits heat loss by reducing the surface to air ratio of each pup (Alberts, 1978), delayed the onset of UV production when pups were faced with a steadily decreasing ambient temperature (Blumberg, Efimova, & Alberts, 1992a). Conversely, increasing metabolic demands by exposing Sprague-Dawley pups to ambient temperatures falling above (i.e., 37°C-41.25°C) the thermoneutral zone (34°C-36°C) increased the amount of time spent calling (Blumberg, et al., 1992a). This latter effect was time-dependent and did not become apparent until the 4th minute of the 6-minute test session. It will be recalled that Allin & Banks (1971) had previously reported that UV rates did not increase when pups were tested at 40°C. However, the test session in their experiment was limited to only 4.5 minutes, and thus may not have been long enough to detect potential changes in UV rates. Taken together, these data show that experimental

manipulations that modify metabolic demands in rat pups alter UVs in a bi-directional manner.

Blumberg et al. (1992a) also demonstrated that in isolated 8- to 9-day-old pups, UV production varied according to relatively minor changes in the ambient temperature, and conformed to a U-shaped distribution. Consistent with previous research (Allin & Banks, 1971), minimal calling took place when pups were isolated within the thermoneutral range (i.e., 34°C-35.25°C). In contrast, calling occurred within temperature ranges falling either below (24.5°C-32.5°C) or above (i.e., 37°C-41.25°C) the thermoneutral range. The finding that UVs were produced at all when pups were tested within the thermoneutral range appears to be incompatible with the hypothesis that UVs are produced as a result of metabolic demands associated with the invocation of thermoregulatory processes. However, Blumberg et al. suggested the possibility that UVs produced under such circumstances result from heat loss and tactile stimulation during transfer of the pup from the homecage to the test apparatus. In support of this hypothesis, they noted that UVs produced under conditions of thermoneutrality dissipated within the first few minutes of testing (as opposed to UVs produced at 20°C, which persisted for the entire 30-minute test session). Subsequent research demonstrated that minimisation of heat loss and tactile stimulation during pup transfer resulted in a significant decrease in UV production during the initial stages of testing at 35°C (Blumberg, Efimova, & Alberts, 1992b). These findings may account for Allin and Bank's (1971) finding that 10- to 12-day-old,

isolated pups emitted UVs when tested within the thermoneutral range. Although Blumberg et al. (1992a,b) did not include measurement of respiration frequency, oxygen consumption, or BAT activation, their data indirectly support a link between ambient temperature and UV production during pup isolation.

Subsequent research by Blumberg and colleagues led to a further refinement of their hypothesis that UVs result directly from physiological changes associated with thermogenesis. When 7- to 9-day-old Sprague-Dawley pups were isolated and faced with moderate cold exposure (i.e., 30°C), subjects were able to thermoregulate successfully (as evidenced by BAT activation, increased oxygen consumption, and the ability to maintain body temperature values at high levels). Under such conditions, UV production remained essentially non-existent. A different pattern emerged, however, when pups were exposed to extreme (i.e., 21°C) reductions in ambient temperature. Under such conditions, BAT activation and increased oxygen consumption failed to maintain body temperature (as evidenced by an inability to maintain interscapular temperature). Under these extreme conditions, UV production increased significantly (Blumberg & Stolba, 1996).

Taken together, these findings demonstrate that there is not necessarily a 1:1 relationship between the initiation of thermoregulatory functions and UV production, as previously reported by Blumberg & Alberts (1990). Rather, it would appear that when faced with moderate cold exposure pups are able to invoke thermoregulatory functions that are not associated with UV production.

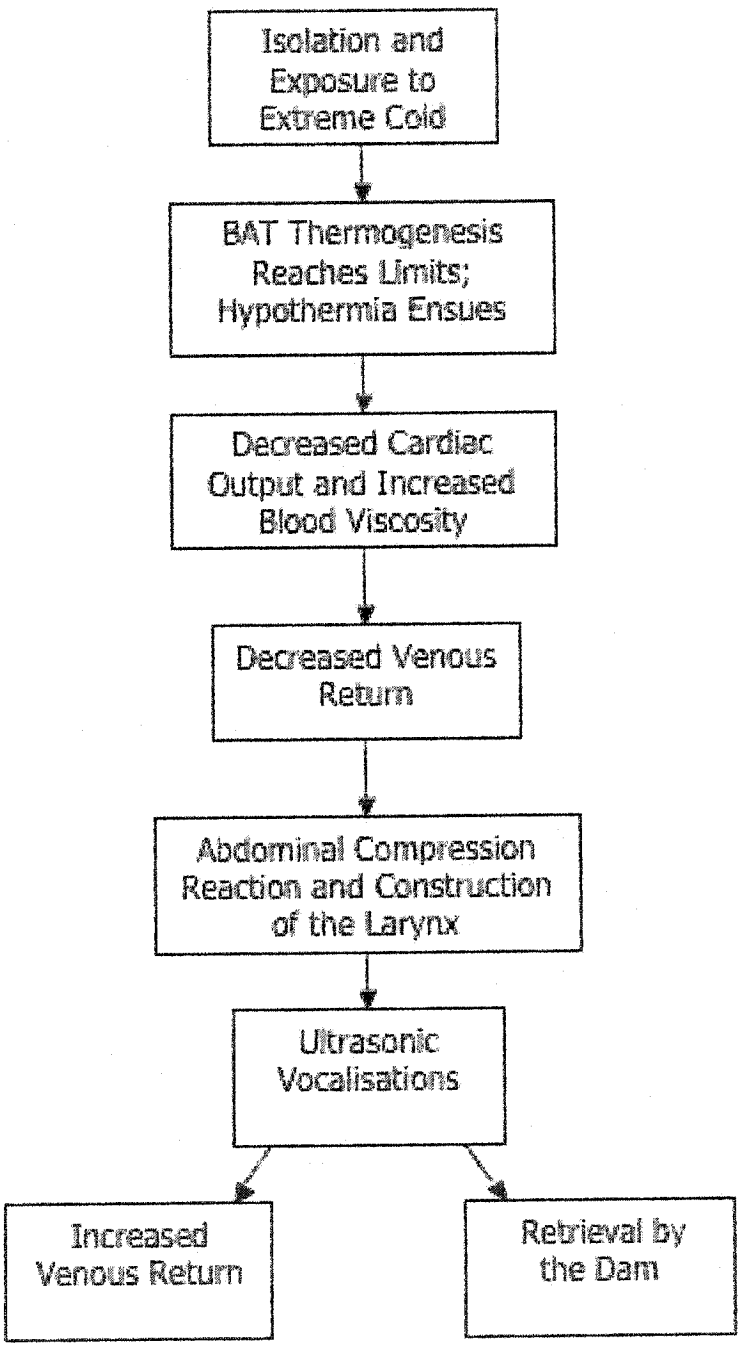
However, under relatively more extreme temperature conditions, UVs occur at the same time as when the pups are faced with ever-increasing metabolic demands. Blumberg, Sokoloff, & Kirby (1997) subsequently demonstrated that under conditions of moderate cold exposure (i.e., when BAT thermogenesis was effective) cardiac rates in Sprague-Dawley pups remained stable. However, extreme cold exposure (and thus when BAT thermogenesis failed and UVs were produced) was associated with a significant decrease in cardiac rate, resulting in a state of bradycardia. BAT thermogenesis was also shown to exert a direct modulatory influence on cardiac rate in Sprague-Dawley rat pups (Blumberg et al., 1997; Sokoloff, Kirby, & Blumberg, 1998). These data led to the hypothesis that UV production might somehow result from a pup's attempt to maintain a stable cardiac rate under conditions of extreme cold exposure.

The importance of cardiac rate for a rat pup's overall cardiovascular system integrity was reviewed by Blumberg and Sokoloff (2001). Briefly, cardiac rate and stroke volume represent the two major factors responsible for cardiac output. During infancy, mammals are limited in their ability to increase stroke volume, and as such, decreased cardiac rate is essentially synonymous with decreased cardiac output. Decreased cardiac output, in turn, results in decreased venous return, and subsequent venous pooling. Under these conditions, general cardiovascular function is threatened. Blumberg, Sokoloff, and Kent (1999) demonstrated that extreme cold exposure also results in increased blood viscosity in 8-day-old Sprague-Dawley rat pups. Increased blood viscosity also

contributes to diminished venous return. In order to compensate for decreased venous return and thus protect the cardiovascular system, a reflexive physiological manoeuvre is elicited, the abdominal compression reaction (ACR; Kirby & Blumberg, 1998). The ACR involves contraction of the abdominal muscles, which forces blood back to the heart, resulting in increased venous return. When abdominal muscles are contracted during the ACR, pulsatile increases in intra-abdominal pressure are also observed, and it is at this time that UVs are produced (Kirby & Blumberg, 1998). Concomitant constriction of the laryngeal plates would serve to further increase intra-abdominal pressure, thus maximising return of blood to the heart. Figure 2 is modified from Blumberg et al. (1999) and Blumberg and Sokoloff (2001), and presents the chain of physiological events hypothesised to lead to UV production by an isolated and cold pup.

If the ACR hypothesis of UV production is correct, then non-thermal stimuli that provoke the ACR should also cause an increase in UV production. Conversely, attenuation of the ACR should be accompanied by a reduction in UV rates. Administration of clonidine, an agonist at the α-2 adrenoreceptor receptor, increases UV production in isolated rat pups (Gardner, 1985a; Hansen, 1993; Hard, Engel, & Lindh, 1988; Kehoe & Harris, 1989, Nazarian, Krall, Osburn, & McDougall, 2001; van der Poel, Molewijk, Mos, & Olivier, 1991), and increased calling has been demonstrated to occur concurrently with the induction of bradycardia in Sprague-Dawley pups (Blumberg, Kreber, Sokoloff, & Kent, 2000;

Figure 2: Sequence of Physiological Events Leading to the Production of Ultrasonic Vocalizations According to the ACR Hypothesis.



Blumberg, Sokoloff, & Kent, 2000; Sokoloff, Blumberg, Mendella, & Brown, 1997). Pre-treatment with prenalterol, a ß-1 adrenoreceptor agonist, which increases cardiac rate, attenuated both the cardiac effects of clonidine and the increase in UV production typically observed following clonidine administration (Blumberg, Kreber, et al., 2000). Finally, sodium nitroprusside and chlorisondamine, like clonidine, are both antihypertensive agents that are associated with decreased venous return, and stimulate UV production under thermoneutral conditions in 15-day-old rats. Conversely hydralazine, an antihypertensive associated with minimal effects on venous return, did not elicit a significant increase in calling amongst Sprague-Dawley pups (Blumberg, Sokoloff, Kirby, Knoot, & Lewis, 2002).

There appears to be sufficient evidence to conclude that thermal factors play an important role in the modulation of isolation-induced UVs, most notably in younger pups, when thermoregulatory abilities are primitive. In older pups, UVs can still be elicited by isolation at ambient temperatures that overwhelm the pup's developing thermoregulatory abilities (i.e., 2°C). These findings would suggest that thermal cues represent primary eliciting factors early in the developmental phase, but then become less salient as pups become increasingly able to cope with low ambient temperatures. In terms of the physiological mechanisms underlying UV production, the data suggest that cardiorespiratory factors are particularly relevant. However, non-thermal factors appear to contribute to UV production to some degree, as evidenced by the fact that 10- to

12-day old pups show an increase in call rates even when tested at thermoneutral temperatures.

1.1.3.2. Isolation-Induced UVs as a Form of Social Communication: Disruption of the Attachment Bond Through the Removal of Multiple Sensory Cues

In contrast to the claims that UVs are merely acoustic by-products of the pup's attempt to cope physiologically with changing thermal demands following isolation, other researchers have suggested that isolation-induced UVs represent the behavioural manifestation of distress or anxiety. Indeed, such a hypothesis is not novel; Zippelius and Schleidt (1956) referred to handling-induced and isolation-induced UVs as "distress" vocalizations, a descriptor that could reasonably imply a link between UVs and the pup's underlying affective state, and as previously noted, Okon (1971, 1972) referred to pup UVs as a way of "calling for help". Proponents of this interpretation concede that thermal cues do indeed play an important role in the elicitation of UVs following separation of a pup from the dam and littermates. However, it is argued that non-thermal (e.g., olfactory and tactile) cues also play an important role in the elicitation of UVs. Emphasis is thus placed on the overall matrix of sensory cues that is hypothesised to underlie the social attachment between the dam and its pups, and how the sudden and simultaneous removal of such cues during isolation stimulates UV production. Each of these different types of cues acts on discrete sensory pathways and is mediated by diverse neurochemical systems, which in

turn modulate the behavioural (including vocal) response of the pup to isolation (Hofer, 1994, 1996, 2002).

In addition to examining how non-thermal cues contribute to isolation-induced UV production, emphasis has also been placed on the fact that the relative contribution of different sensory cues to the production of isolation-induced UVs varies as a function of the age of the pup at testing (Hofer 1994, 1996, 2002; Shair, Brunelli, Masmela, Boone, & Hofer, 2003). As previously noted, during the early phase of the developmental period (roughly the first postnatal week) thermal and non-thermal modulate UVs. After the first postnatal week, non-thermal cues become more salient in terms of their ability to modulate isolation-induced UVs, and thermal cues exert less influence (Hofer, 1996). This interpretation is in keeping with the notion that as pups develop homeothermic abilities with increasing age, the threat of low ambient temperature diminishes. The contribution of non-thermal factors in the production of isolation-induced UVs will now be considered.

Rat pups are able to discriminate between olfactory cues early during the course of postnatal development (Conely & Bell, 1978; Oswalt & Meier, 1975; Polan & Hofer, 1998; Salas, Schapiro, & Guzman-Flores, 1970; Tobach, Rouger, & Schneirla, 1967), and pups use olfactory cues to help detect (Schapiro & Salas, 1970) and locate (Leon & Moltz, 1972) the dam. However, selective removal of olfactory cues is not sufficient to prevent isolation-induced UVs. Hofer and Shair (1980, 1991a) and Shair, Masmela, and Hofer (1999) reported that 12- to 14-

day-old Wistar rat pups, rendered anosmic following nasal infusion with zinc sulphide ($ZnSO_4$), did not differ from controls in terms of their increased UV response to isolation at room temperature. This finding suggests that the removal of other sensory cues (e.g., tactile) was sufficient to evoke increased calling amongst the isolated pups. This is not to say that olfactory cues do not modify isolation-induced UVs, but rather that the vocal response of isolated pups is likely mediated by a combination of tactile, olfactory, and thermal cues (Hofer, Brunelli, & Shair, 1993b).

A role for olfactory cues in the modulation of UVs was demonstrated by Oswalt & Meier (1975), who reported that 5- and 13-day-old pups isolated at room temperature emitted fewer UVs when placed on a cloth above a dish containing soiled bedding from the homecage compared to when they were placed on a cloth positioned above a dish of clean bedding. The reduction in isolation-induced UV rates associated with exposure to soiled homecage bedding has also been demonstrated by other investigators (Conely & Bell, 1978; Richardson & Defina, 1998), and while negative results have also been reported (Hofer & Shair 1980), these authors suggested that a procedural difference between studies might have accounted for their findings. Specifically, whereas Oswalt and Meier (1975) and Conely and Bell (1978) placed the soiled bedding directly beneath the isolated pup, Hofer and Shair (1980) suspended a mesh container of soiled bedding above the isolated pup. Hofer & Shair (1980) suggested that suspending the bedding above the pup might have failed to

provide the pups with a familiar localising source for the odour. UVs might have been unaffected because the odour did not originate from beneath the pup (as would odour from litter when the pup is in its homecage). Finally, strain differences might have contributed to the discrepant findings to some degree. Sprague-Dawley rats were used in the three studies reporting positive findings (i.e., Oswalt & Meier, 1975, Conely & Bell, 1978, Richardson & Defina, 1998), whereas Wistar pups were used by Hofer & Shair (1980).

In addition to the effects of nest odours on UVs, Conely and Bell (1978) reported that isolated 3-, 5-, 7-, and 9-day-old pups exposed to the odour of an unfamiliar adult male also responded with reduced UV rates, a finding that has proved to be a reliable behavioural response by Sprague-Dawley pups (Takahashi, 1992a,b, 1994). Adult male rats pose a significant threat to pups, and it is not uncommon for the male to kill unknown pups if they are encountered (Brown, 1986). Exposure to an adult male rat has also been shown to affect a number of additional behavioural and physiological responses in the pup (e.g., increased immobility and urination, and activation of the hypothalamic-pituitary-adrenal axis) consistent with a stress response (Takahashi 1992a,b, 1994; Takahashi & Kim, 1995). As such, the decrease in UVs (and other behavioural and physiological changes) following presentation of an adult male has been termed as "behavioural inhibition" (Takahashi, 1994), representative of a fear response by the pup.

Presentation of another rat to an isolated pup has been noted to exert significant effects on UVs emitted by isolated pups, and olfactory cues are also involved in these situations. Specifically, when 12- to 14-day-old Wistar pups are isolated at room temperature and then presented with the anesthetized dam, the pups respond with a decrease in UV rates (Hofer, Brunelli, & Shair, 1993b; Hofer & Shair, 1978, 1987; Shair, Masmela, & Hofer, 1999). This effect has been termed as "contact quieting" (Hofer, Masmela, Brunelli, & Shair, 1998). A similar response has been detected following presentation of a single anesthetized littermate in Wistar pups as young as 3 days old (Carden & Hofer, 1992; Hofer and Shair, 1978) or presentation of a group of anesthetized littermates (Hofer & Shair, 1980). These effects appear to be independent of prior social experience, as contact quieting has been recorded from Wistar pups that were raised deprived of social interactions from as early on as the day of birth (Hofer & Shair, 1987; Hofer, Shair, & Murowchick, 1989).

Introduction of an anesthetized unfamiliar male also induces a decrease in UVs in Wistar pups (Shair, Masmela, & Hofer, 1999). This finding is consistent with the quieting effects of unfamiliar male odours on isolation-induced UVs, and the view that cues associated with an unfamiliar male provokes an inhibitory, or fear response in the isolated pup. Consistent with the view that pup UVs serve as directional cues to facilitate retrieval of the isolated pup by the dam, then decreased calling in the presence of a potentially threatening adult male represents an adaptive behavioural response on the part of the pup to avoid

detection by a potential predator. In contrast, exposure to the dam or littermates is associated with high levels of physical contact between the previously isolated pup and the anesthetized subject (Hofer & Shair, 1978, 1980), which suggests that the experience is affiliative (positive), rather than aversive.

It is important to note that both *decreased* UVs in the presence of an unfamiliar male, and *increased* UVs during isolation both are conceptualised as representing the behavioural manifestation of negative emotional states (fear or anxiety). This apparent contradiction can be reconciled if consideration is given to ethological factors associated with each of these conditions. From an evolutionary point of view, a pup will increase its likelihood of survival if it decreases its rate of calling (and hence cues associated with its location) when it is isolated and a potential predator is near. Conversely, increasing call rates during isolation (providing that no potential predator is near) will also increase the pup's chance of survival by helping the dam locate the pup so she can bring it back to the safety of the nest.

When considering the potential role for olfactory cues in mediating the pup's response to these different social stimuli, it is important to note that decreased calling in response to a littermate is absent amongst isolated Wistar pups previously rendered anosmic following nasal perfusion with $ZnSO_4$. (Hofer & Shair, 1980). These data would appear to suggest that olfactory cues are essential in mediating the UV response under the above noted circumstances.

However, Hofer and Shair (1980) found that isolated pups exhibited reduced UVs when presented with a dead littermate that had been thoroughly washed with acetone, alcohol, and water (thus removing any familiar olfactory cues). In addition, exposure to the natural odours of a dead littermate did not elicit a reduction in isolation-induced UVs. These findings appear to contradict the data obtained from anosmic pups, and suggest that olfactory cues associated with a littermate are in fact not necessary in order to induce a decrease in calling.

Hofer and Shair (1980) postulated that the failure to provoke a decrease in UVs in anosmic pups in their study might have stemmed from a procedural effect associated with nasal perfusion. Specifically, following nasal perfusion, both control (infused with saline) and experimental pups recovered overnight in isolation. The authors suggested the recovery in isolation might have affected the subjects' subsequent UV response to the littermate. In support of this interpretation they noted that while the control group's UV rates were indeed reduced by introducing the littermate, this effect was attenuated (i.e., UVs were reduced only 50% amongst control subjects, compared to a 91% reduction for pups that had been housed in their homecage with littermates prior to isolation testing). Furthermore, Hofer and Shair (1991a) reported that anosmic Wistar pups housed overnight with littermates (but without the dam) did indeed exhibit a standard companion response when isolated and then presented with an anesthetized littermate the next day. Shair and colleagues (1999) employed the same group housing procedure (i.e., with littermates but without the dam) for

pups recovering from nasal perfusion. Anosmic pups in this study failed to exhibit decreased UVs when presented with either the anesthetized dam or an unfamiliar male. It is possible that prior housing conditions (i.e., in isolation vs. with littermates) interact with the type of stimulus subsequently used (i.e., a littermate versus the dam or an unfamiliar male) to modulate the anosmic pup's UV response when presented with another rat.

Tactile cues have also been implicated in the modulation of isolation-induced UVs. In the Oswalt and Meier (1975) study, 3- to 13-day-old pups vocalised more when placed in an empty dish compared to when placed in the same type of dish that was covered with clean bedding. Ambient temperature was controlled across the two conditions, thus ruling out the contribution of thermal cues. The observed effect could also not be accounted for exclusively by olfactory cues. When tactile cues were held constant by isolating pups on a cloth positioned above either the empty dish or the dish containing clean bedding, vocalizations remained significantly different only among 3-day-old pups. As such, it would appear that in the older pups, the elevated UV rate associated with placement on the empty dish (versus on clean bedding) was associated with tactile, as opposed to olfactory cues.

It will be recalled that an isolated pup responds to presentation of an anesthetized littermate with a reduction in UV rates (Hofer & Shair 1978, 1980). In addition, Hofer and Shair (1978) found that when isolated with an anesthetized littermate, pups spent most of the 6-minute test period in direct

contact with the littermate. However, the post-test body temperatures of these pups did not differ significantly compared to the post-test body temperatures of the same pups following isolation without the littermate. Thus it would appear that thermal factors associated with the anesthetized pup could not exclusively account for the observed reductions in UVs (Hofer, 1978). In addition, isolated pups do not exhibit decreased UV rates when presented with a small, rubber, clay-filled model, heated to 35°C-36°C (Hofer & Shair 1978, 1980). Finally, Hofer et al. (1993b) found that dams cooled to 10°C were just as effective as dams possessing normothermic (i.e., 35°C) body temperatures, in terms of the dams' ability to reduce isolation-induced UVs. Thermal properties of the dam were found to play a modulatory role, however, as a negative correlation was detected between dam flank temperature and UV rates. Taken together, the data from the dam and littermate studies indicated that thermal cues alone couldn't account for reductions in UV rates when isolated pups are presented with another rat. Tactile and olfactory cues are also clearly involved.

In order to investigate the relative contribution of different sensory cues in modulating UVs, Hofer and Shair (1980) presented isolated, 2-week-old pups with an anesthetized littermate or one of six different inanimate stimuli. Each of the stimuli possessed a different combination of sensory cues (thermal, tactile, olfactory), with each cue designed to mimic an aspect of the anesthetized littermate. For example, relatively basic stimuli possessed only one cue (e.g., nest odour only, derived from nest shavings suspended above the test

compartment) or two cues (contour and thermal cues, derived from a warm model consisting of clay encased in the finger of a rubber glove and heated to 35°C). In contrast, relatively more complex stimuli possessed an increasing number of familiar sensory cues (e.g., a dead and heated littermate, possessing all of the sensory cues of an anesthetized littermate except for the absence of respiration). Isolated pups did not respond with decreased UV rates when presented with either the warm model or the nest odour. However, as the stimuli became increasingly complex in terms of the number of sensory cues provided, the pup's UV rates decreased in a graded fashion; the more sensory cues provided to mimic the constellation of cues provided by an anesthetized littermate, the greater the reduction in UV rates. In addition, a significant correlation was noted between the amount of direct physical contact elicited by each stimulus and the degree to which each stimulus reduced UVs in the isolated pup. A similar correlation has also been reported when an anesthetized dam is used as the stimulus (Hofer et al., 1989). Hofer and Shair (1980) concluded that the reduction in UV rates observed when an isolated pup is presented with an anesthetized littermate does not result from exposure to a single sensory cue. Conversely, when a pup faces isolation in an unfamiliar environment, its increased UV response is likely not due to the sudden removal of any one type of sensory cue, but rather a combination of thermal, olfactory, and sensory cues.

1.1.3.3. Direct Challenges to Blumberg's Acoustic-By-Product Hypotheses

It will be recalled that Blumberg and colleagues presented two hypotheses regarding how isolation-induced UVs might represent acoustic by-products stemming from attempts by the pup to physiologically cope with thermal demands. In the case of both hypotheses, physiological responses to decreased temperature co-occur with laryngeal braking, resulting in UV production. Within the framework of the first hypothesis, UVs are produced in order to help meet the metabolic demands associated with BAT thermogenesis (Blumberg & Alberts, 1990), and laryngeal braking serves to meet increased demands for oxygen. The second hypothesis states that UVs result from the ACR, which in turn is produced in order to increase venous return (Kirby & Blumberg, 1998). Laryngeal braking increases intra-abdominal pressure, thus maximising the propulsion of blood back to the heart.

While the data from studies assessing the potential role for non-thermal factors in the production of isolation-induced UVs do not support the notion that increased calling is solely in response to thermal cues, these studies do not directly address either of the thermally-based hypotheses put forward by Blumberg and colleagues. However, Hofer and Shair (1991b) found that 12- to 13-day-old Wistar pups emitted UVs when isolated in a cold environment, even when their ability to initiate BAT thermogenesis was blocked by pre-treatment with sympathetic ganglia-blocking agent hexamethonium. In addition, prevention of BAT activation by nutrient deprivation or by surgical excision of interscapular

fat deposits also failed to diminish the UV response to isolation. Conversely, pharmacological activation of BAT following administration of norepinephrine (NE) failed to provoke UVs in a thermoneutral environment. Of note, pups emitted high rates of vocalisation at the conclusion of testing when they were handled by the experimenter in a manner previously documented to stimulate UV production (thus demonstrating that the pups' lack of UV production during BAT activation was not due to an unspecified effect of NE treatment).

BAT activation and increased UVs in response to isolation thus appear to represent independent physiological and behavioural responses to cold. These responses both serve adaptive functions (to increase central warming in the case of BAT, and to elicit maternal retrieval in the case of UVs), but the occurrence of one does not necessarily evoke the occurrence of the other (Hofer & Shair, 1991b). In a separate series of studies, Hofer and Shair (1993) found that neither laryngeal denervation nor tracheostomy had any effect on rates of cooling or BAT thermogenesis among 9- to 10-day-old Wistar pups following isolation at room temperature, a finding that casts further doubt on the hypothesis that laryngeal braking (and associated UVs) serve to facilitate the increased metabolic demands associated with BAT activation.

Recently, Shair & Jasper (2003) addressed the ACR hypothesis directly by experimentally inducing the ACR. As previously noted, pre-treatment with sodium nitroprusside, which induces decreased venous return, induced UVs in 15-day-old Sprague-Dawley pups (Blumberg et al., 2002). However, Shair and Jasper (2003)

found that pre-treatment with the same compound failed to increase UV rates in 15-day-old pups from two other strains, the Wistar or N:NIH lines. Of note, Sokoloff and Alberts (2002) reported that strain differences (between Sprague-Dawley and Long Evans rats) exist with respect to the cardiovascular response to sodium nitroprusside treatment. As such, it is possible that the ACR underlies UV production in a strain-specific manner.

Shair and Jasper (2003) also reported that blood withdrawal (another procedure which induces the ACR response) also failed to increase UV rates in 12- to 15-day-old Wistar pups. Finally, the experimental induction of increased venous return (i.e., thus accomplishing the hypothesized goal of the ACR and UVs) via intravenous treatment with a dextrose and water mixture, or blood, both failed to reduce calling amongst isolated 12-day-old Wistar pups. Taken together, these findings indicate that that the ACR is not sufficient (at least in Wistar and N:NIH pups) or necessary (at least in Wistar pups) to increase UVs.

A review of the data regarding the contribution of olfactory and tactile cues to the production of UVs, and the challenges to the BAT and ACR hypotheses, indicates that a purely temperature-based explanation for why pups produce UVs when isolated is likely not tenable. However, support for the hypotheses put forth by Blumberg and his colleagues does appear to exist when pups are tested under a specific set of environmental conditions, during recovery from extreme hypothermia (Hofer and Shair, 1992, 1993). In this situation, pups that are essentially unconscious while re-warming respond with increased UVs.

Laryngeal nerve transection or tracheostomy under these circumstances slowed the re-warming process (recall that these procedures had no effect on thermogenesis in pups that were isolated at room temperature). UVs produced during recovery from hypothermia thus appear to be part of a co-ordinated physiological response to increased thermoregulatory demands, consistent with the view put forth in 1990 by Blumberg and Alberts (Hofer and Shair, 1992, 1993).

1.1.4. The Neuropharmacology of UVs: Effects of Anxiolytic and Anxiogenic Drugs

Given that UVs are influenced by a number of sensory cues, it is not surprising to find that these calls are affected by a wide range of pharmacological compounds, which act on discrete neurochemical systems (Hofer, 1996; Insel & Winslow, 1991a; Miczek, Tornatzky, & Vivian, 1991; Winslow & Insel, 1991a,b). In this respect, activation of the γ-aminobutyric acid (GABA) (Olivier, Molewijk, van Oorschot, van der Heyden, Ronken, & Mos, 1998), excitatory amino acid (EAA) (Kehne, McClosky, Baron, Chi, Harrison, Whitten, & Palfreyman, 1991; Podhorna & Brown, 2000a; Winslow, Insel, Trullas, & Skolnick, 1990), or cholinergic (Kehoe, Callahan, Daigle, Mallinson, & Brudzynski, 2001) systems, exerts significant effects on UVs. Monoaminergic involvement in UV modulation has been established for drugs acting on serotonin (5-HT) (Olivier, Molewijk, van der Heyden, van Oorschot, Ronken, Mos, & Miczek, 1998), NE (Hård, Engel, Lindh, 1988; Kehoe & Harris, 1989; Nazarian et al., 2001; van

der Poel et al., 1991), and dopamine (DA) systems (Bartoszyk, 1998; Cuomo, Cagiano, Renna, De Salvia, & Racagni, 1987; Dastur, McGregor, & Brown, 1999). In addition, there is ample data demonstrating that opioid peptides modulate UVs (Carden, Barr, & Hofer, 1991; Carden, Davichi, & Hofer, 1994; Carden, Hernandez, & Hofer, 1996; Carden & Hofer, 1990, 1991; Kehoe & Boylan, 1994), and that other peptides, including corticotrophin releasing factor (CRF) (Harvey & Hennesey, 1995; Insel & Harbaugh, 1989; Kehne, Coverdale, McCloskey, Hoffman, & Cassella, 2000), vasopressin (Winslow & Insel 1993), and oxcytocin (Insel & Winslow, 1991b) also affect calling in isolated pups. Finally, in some studies, manipulations involving cholecystokinin (CCK) have been shown to affect UV production when pups are either isolated or when they are in contact with the dam (Blass & Shide, 1993; Rex, Barth, Voight, Domeney, & Fink, 1994; Weller & Blass, 1988; Weller & Dubson, 1998; Weller & Gispan, 2000). The effects of CCK on UVs will be detailed in a subsequent section.

When evaluating the data from studies regarding the neuropharmacological basis of UVs, it is important to note that widespread inter-study variability exists. For example, common differences across studies include: strain of rats tested, age of subjects at testing, dose and route of drug administration, duration of pre-treatment time prior to testing, ambient temperature during testing, duration of the test session, and method of quantifying UV data (e.g., mean rates, median rates, percentage change, transformed scores). In addition to changes in UVs, some studies have included

measurement and analyses of motor activity levels and/or body temperature, in order to determine whether the effects of a given drug are specific to UVs. In other studies, only UV rates have been assessed. Due to these many methodological differences, cross-study comparisons are difficult. However, many pharmacological compounds that possess anxiolytic properties in humans and other animals have been found to reduce isolation-induced UVs. In this respect, examination of the effects of benzodiazepines, partial agonists of the 5-HT_{1A} receptor subtype, and selective serotonin re-uptake inhibitors (SSRIs) are particularly relevant, in order to validate isolation-induced UVs as a behavioural index of anxiety.

Benzodiazepine agonists have long been used for the clinical treatment of anxiety disorders (including generalized anxiety disorder, panic disorder, and social phobia), despite the fact that many such compounds possess an adverse side effect profile (Bateson, 2002; Salzman, Goldenberg, Bruce, & Keller, 2001; Sheehan, 2002; Uhlenhuth, Balter, Ban, & Yang, 1999). Given the long history of benzodiazepine use in clinical settings, animal models of anxiety are typically validated by assessing the degree to which the experimental behaviour in question responds to pre-treatment with these compounds (Martin, 1998). In this respect, full and partial benzodiazepine agonists such as diazepam, chlordiazepoxide, alprazolam, oxazepam, zolpidem, bretazenil, and alpidem decrease isolation-induced UVs. These effects can be achieved without inducing significant changes in body temperature or motor activity (although in many

cases, the degree to which motor activity is also affected is dose dependent) (Carden & Hofer, 1990; Gardner, 1985a,b; Gardner & Budhram, 1987; Insel, Hill, & Mayor, 1986; Olivier et al., 1998; Podhorna & Brown, 2000a; Vivian, Barros, Manitiu, & Miczek, 1997).

Allopregnanolone, a neurosteroid that acts at the $GABA_A$ receptor site, also reduces UVs (Vivian et al., 1997; Zimmerberg, Brunelli, & Hofer, 1994). Benzodiazepine antagonists have been shown to block the UV-reducing effects of agonists, and in some cases, actually reduce UVs intrinsically (Carden & Hofer, 1990; Olivier et al., 1998). This latter finding has led to the hypothesis that stress in the form of isolation leads to the release of an endogenous, anxiogenic compound that normally binds with benzodiazepine receptors. Pre-treatment with a benzodiazepine antagonist would prevent the endogenous ligand from binding to the benzodiazepine receptor, which in turn would result in a diminished UV response to isolation (Insel, Hill, & Mayor, 1989; Olivier et al.). This hypothesis is supported by the results from receptor-binding studies, which have revealed that isolation is associated with decreased benzodiazepine receptor availability in several brain regions (i.e., the frontal cortex, hippocampus, and superior and inferior colliculi) in 10-day-old rat pups (Insel, 1989; Insel, Gelhard, & Miller, 1989).

Partial $5\text{-}HT_{1A}$ agonists are effective in the treatment of generalised anxiety disorder (Sramek, Zarotshy, & Cutler, 2002), and these compounds (e.g., buspirone and isapirone) also reduce UV rates (Kehne et al., 1991; Olivier et al.,

1998). In addition, full 5-HT_{1A} receptor agonists such as ((+/-)-8-hydroxy-2-(di-N-propylamino)tetralin) (8-OH-DPAT) and flesinoxan also reduce UVs (Hård & Engel, 1988; Johansson-Wallsten, Berg, & Myerson, 1993; Joyce & Carden, 1999; Mendella et al., 1997; Olivier et al., 1998; Winslow & Insel, 1990, 1991c). The effects of partial and full 5-HT_{1A} agonists are blocked by pre-treatment with 5-HT_{1A} antagonists, and can be induced without affecting motor activity, negative geotaxis or core body temperature. However, conflicting data have been also been reported in this respect, and the selectivity of the observed UV effects appear to be dependent on the dose employed and the age of the rat pups at testing (Hård & Engel, 1988; Johansson-Wallsten et al., 1993; Olivier et al., 1998; Winslow & Insel, 1991c). Of note, when 5-HT_{1A} agonists have been shown to affect body temperature in conjunction with reducing UVs, the effect has typically been to decrease body temperature. This dissociation between the effects on UVs and body temperature would suggest that the effects of 5-HT_{1A} agonists on UVs are not secondary to thermal changes, as a decrease in body temperature would be expected to be associated with increased, not decreased calling.

Selective serotonin reuptake inhibitors (SSRIs) have been shown to be effective in the treatment of number of anxiety disorders, including generalized anxiety disorder (paroxetine), panic disorder (fluvoxamine, fluoxetine, citalopram, sertraline, paroxetine), post traumatic stress disorder (fluvoxamine, fluoxetine, paroxetine, sertraline), obsessive-compulsive disorder (fluvoxamine,

fluoxetine, citalopram), and social phobia (fluvoxamine, paroxetine) (Vaswani, Linda, & Ramesh, 2003; Zohar & Westenberg, 2000). These compounds have been reported to exert specific, inhibitory effects on isolation-induced UVs, (Mos & Olivier, 1988; Olivier et al., 1998; Winslow & Insel, 1990). The mixed 5-HT/NE reuptake inhibitor imipramine, which is used in the treatment of generalised anxiety disorder and panic disorder (Kapczinski, Lima, Souza, & Schmitt, 2003; Sramek et al., 2002) selectively decreases UVs in a dose dependent manner (Olivier et al.). Flibanserin, a putative antidepressant/anxiolytic agent that acts as mixed 5-HT_{1A} agonist/5-HT_{2A} antagonist, also selectively decreases isolation-induced UVs (Podhorna & Brown, 2000b). Finally, tianeptine, which increases the 5-HT reuptake process, is used an antidepressant and effectively treats anxiety that often co-occurs with depression (Wagstaff, Ormrod, & Spencer, 2001). While this compound inhibits UVs, it also significantly impairs motor activity (Olivier et al.).

In contrast to the effects of anxiolytics on UV rates, established anxiogenic compounds tend to increase UV rates. Specifically, Insel et al. (1986) reported that the anxiogenic GABA-ergic compound pentylenetetrazol significantly increased UV rates in an anxioselective manner. The benzodiazepine inverse agonists FG-7412 (N′-methyl-β-carboline-3-carboxamide), and β-CCE (methyl-β-carboline-3-carboxylate), which are also purported to possess anxiogenic effects, also increased isolation-induced UV rates. However, the effects of these latter compounds were not as robust compared to the anxiogenic

effect of pentylenetetrazol. Moreover, negative results with inverse agonists have also been reported. Specifically, FG-7142 and the inverse agonist DMCM (6,7-dimethyl-4-ethyl-β-carboline-3-carboxylate) have been reported to have no significant effects on UVs (Olivier et al., 1998). Findings with FG-7142 may however, be dose dependent, given that the highest dose tested by Oliver and colleagues was 10mg/kg, and the dose found to elicit UVs by Insel et al. was 25mg/kg.

There is a significant exception to the general rule that anxiolytic compounds also reduce isolation-induced UVs. As noted in a previous section, the α-2 adrenoreceptor receptor agonist clonidine has consistently been reported to increase UVs (Gardner, 1985; Hansen, 1993; Hård et al., 1988; Kehoe & Harris, 1989, Nazarian et al., 2001; van der Poel et al., 1991), despite the fact that clonidine is anxiolytic in humans facing alcohol withdrawal symptoms or amongst individuals with panic disorder (Hoehn-Saric, Merchant, Keyser, Smith, 1981). This inconsistency has been attributed to central maturational changes in the developing noradrenergic system of the rat. Consistent with this interpretation, a shift in the effects of clonidine on UVs has been noted, whereby after 17 days of age, clonidine no longer induces an increase in calling (Hård et al.; Kehoe & Harris, 1989). The UV data pertaining to clonidine underscore the importance of considering neurodevelopmental variables when evaluating the effects of different drugs on UV rates in isolated pups.

In summary, the above data indicate that numerous neurochemical systems are involved in the modulation of UVs. Data assessing the UV effects of GABA-ergic and serotonergic anxiolytic and anxiogenic compounds tend to provide "neuropharmacological validity" to the hypothesis that UVs produced in isolation represent a form of anxiety or distress. However, in light of the data pertaining to the effects of clonidine, the utility of isolation-induced UVs as an animal model might be limited to evaluating drugs that act on specific neurochemical systems.

1.2. Cholecystokinin

1.2.1. An Overview of the Neurobiology of CCK

CCK was first characterized as a gastrointestinal hormone, involved in the regulation of gut motility, pancreatic secretion, and contraction of the gall bladder (see Crawley & Corwin, 1994, for a review of CCK's effects on digestive processes). CCK is derived from the 115 amino acid precursor molecule, prepro-CCK (Deschenes et al., 1984), and numerous biologically active CCK fragments have been identified (Crawley & Corwin, 1994). The discovery of gastrin-like immunoreactivity in the rat brain (Vanderhaeghen, Signeau, & Gepts, 1975) subsequently led to the characterisation of CCK or CCK mRNA in numerous mammalian brain structures including the cerebral cortex, hippocampus, amygdala, septum, olfactory tubercle, olfactory bulb, hypothalamus, thalamus, basal ganglia, and the brainstem (Beinfeld, Meyer, Eskay, Jensen, & Brownstein, 1981; Crawley & Corwin, 1994; Moran & Schwartz, 1994; Sauter & Frick, 1983).

Of the many CCK fragments, the sulphated C-terminal octapeptide CCK-8s (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) is the most abundantly distributed fragment in rat and human brains, and CCK is recognised as the most widely distributed of all the neuropeptides found in the brain (van Megen, Westenberg, den Boer, & Kahn, 1996). An additional biologically active CCK fragment that is relevant for the purposes of the current research is the tetrapeptide fragment, CCK-4 (Trp-Met-Asp-Phe-NH_2).

CCK meets the established criteria for designation as a neurotransmitter. Specifically, it is found (Larsson & Rehfeld, 1979) and synthesized (Golterman, 1985) within neurons, and localised within neuronal terminals (Emson, Lee, & Rehfeld, 1980). CCK is released following depolarization (Iverson, Lee, Gilbert, Hunt, & Emson, 1980), and then inactivated following its release by enzymatic degradation (Koulischer, Moroder, & Deschodt-Lanckman, 1982). CCK receptors have been identified in the periphery and in the brain (see below) (Noble et al., 1999). Finally, exogenous application of CCK to the post-synaptic membrane results in the same effect as that seen following neuronal release of CCK (Bradwejn & de Montigny, 1984). In addition to fulfilling the criteria for a neurotransmitter, CCK exerts a modulatory role on other neurotransmitters. For example, CCK interacts and/or is colocalised with DA (Hökfelt et al., 1980; Hommer, Stoner, Crawley, Paul, & Skirboll, 1986), 5-HT (Bickerdike, Marsden, Dourish, & Fletcher, 1994; Voigt, Sohr, & Fink, 1998), NE (Kendrick, Leng, & Higuchi, 1991), GABA (Bradwejn & de Montigny, 1984; Takeda, Nakata,

Takahashi, Chikuma, & Kato, 1998), EAAs (Ge, Long, & Kilpatrick, 1998; Hökfelt et al., 2002; Yaksh, Furui, Kanawati, & Go, 1987), and opioids (Wiesenfeld-Hallin, Lucas, Alster, Xu, & Hökfelt, 1999). CCK typically exerts an excitatory central effect, although neuronal inhibition has been found when CCK is applied to the nucleus tractus solitarius (Crawley & Corwin, 1994). In addition, while microiontophoretic application of CCK on to midbrain DA neurons has a "typical", excitatory effect, co-administered CCK and DA potentiates the inhibitory effects of DA (Crawley, 1991).

Two CCK receptor subtypes have been identified, which have traditionally been characterised as the CCK-A and CCK-B receptors. Recently, these subtypes have been renamed as the CCK-1 and CCK-2 receptors, respectively (Alexander & Peters, 1998). Both receptors belong to the G-protein coupled receptor family (Dunlop, 1998). CCK-1 receptors are primarily found in the periphery (the "A" designation associated with the original name for this receptor type is derived from "alimentary"), and have a 500- to 1000-fold higher affinity for CCK-8s versus CCK-4 or the unsulphated form of CCK-8, CCK-8us (Crawley & Corwin, 1994). Initially, results of autoradiographic studies revealed a limited number of CCK-1 receptors in the brain, primarily in the nucleus tractus solitarius, area postrema, and the interpeduncular nucleus, (Hill, Campbell, Shaw, & Woodruff, 1987; Hill, Shaw, Dourish, & Woodruff, 1988; Moran, Robinson, Goldrich, & McHugh, 1986). More recent data, however, have revealed that central CCK-1 receptor distribution is more extensive, and this receptor subtype can also be

found in the substantia nigra, ventral tegmental area, periventricular nucleus of the hypothalamus, basal ganglia, and the nucleus accumbens. In addition, considerable inter-species variability exists with respect to CCK-1 receptor distribution in the brain (Bradwejn & Koszycki, 2001; Davidowa, Wetzel, & Henklein, 1997; Mercer & Beart, 1997; Noble et al., 1999).

The CCK-2 receptor subtype displays a high affinity for multiple CCK fragments, (including both CCK-8s and CCK-4), with approximately a 100-fold affinity for CCK-4 over CCK-8s (Crawley & Corwin, 1994). CCK-2 receptors are found to a limited degree in the periphery (i.e., stomach and vagus nerve) but are located in many central sites, including the cerebral cortex, olfactory bulb, nucleus accumbens, caudate nucleus, hippocampus, hypothalamus, amygdala, substantia nigra, ventral tegmental area, dorsal raphe, and cerebellum (see Crawley and Corwin, 1994; Noble et al., 1999, for reviews).

CCK and CCK receptors are present early in development, both in the central nervous system (Hays, Houston, Beinfeld, & Paul, 1981; Pelaprat, Dusart, & Peschanski, 1988), and in the periphery (Brand, 1982; Robinson, Moran, Goldrich, & McHugh, 1987). CCK and CCK receptor ontogeny in the brain occurs almost exclusively during the postnatal period. At postnatal days 1-2, the density of CCK receptors in the rat forebrain is low. However, levels steadily increase in the forebrain, thalamus, and mesencephalon, reaching peak levels between days 12 and 17. Receptor levels then begin to drop until adult levels are reached within 4 weeks postnatally (Hays et al., 1981; Pelaprat et al., 1988). CCK levels

in the brain are essentially undetectable at birth, but rise quickly, peaking at 20-28 days (Beinfeld, Korchak, Nilaver, & O'Dorisio, 1983; Brand, 1982).

In light of the widespread central distribution of both CCK fragments and CCK receptors, and many interactions that exist between CCK and "classical" neurotransmitters (see above), it is not surprising to find that CCK has been implicated in the mediation of number of physiological processes and different aspects of cognitive and behavioural functioning. The former include various aspects of digestion, cardiorespiratory functioning, analgesia, body temperature regulation, and cancer cell proliferation. CCK has also been investigated in terms of its possible involvement in feeding (including eating disorders), sexual and reproductive behaviours, sleep, memory, schizophrenia, depression, drug withdrawal, and anxiety/panic reactions (see Crawley & Corkin, 1994; Daugé & Léna, 1998; Fink, Rex, Voits, & Voigt, 1998; Rotzinger & Vaccarino, 2003; van Megen et al., 1996, for reviews).

1.2.2. Role of CCK in Human Panic

Panic attacks are described in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; American Psychiatric Association, 1994) as bouts of intense fear or discomfort that are accompanied by at least four of 13 somatic or cognitive symptoms. Such attacks develop abruptly and reach peak intensity within 10 minutes. The 13 somatic or cognitive symptoms are palpitations, sweating, trembling or shaking, sensations of shortness of breath or smothering, feeling of choking, chest pain or discomfort, nausea or abdominal distress,

dizziness or lightheadedness, derealisation or depersonalization, fear of losing control or 'going crazy', fear of dying, paresthesias, and chills or hot flashes. Panic attacks occur in three characteristic forms. Unexpected, or uncued attacks occur "out of the blue". Situationally bound, or cued attacks occur almost invariably immediately on exposure to, or in anticipation of, a situational cue or trigger. Finally, situationally predisposed panic attacks are more likely to occur on exposure to a situational cue or trigger (although the individual can be exposed to the cue/trigger without having a panic attack, or the panic attack can occur some time after exposure).

The experience of panic attacks occurs within the context of numerous anxiety disorders, including panic disorder, social phobia, specific phobia, posttraumatic stress disorder, and acute stress disorder. A diagnosis of panic disorder requires the recurrent experience of panic attacks (with at least some of the attacks being uncued). In addition, a month (or more) following at least one panic attack, the individual must experience persistent concern about having more attacks, and/or worry about the implication of the attack or its consequences, and/or experience a significant change in behaviour related to the attacks. Finally, the panic attacks cannot result from the direct physiological effects of a substance or a general medical condition, nor can they be better accounted for by another mental disorder (American Psychiatric Association, 1994).

The neuroanatomical and neuropsychopharmacological basis of panic is complex, and the affective, cognitive, and physiological symptoms of panic have been associated with numerous central sites and neurochemical systems (see Bourin, Baker, & Bradwejn, 1998; Zacharko, Koszycki, Mendella, & Bradwejn, 1995, for reviews). Given the widespread distribution of CCK and CCK receptors within the brain (including in limbic sites and the brainstem), and its colocalisation and interactions with other neurochemicals, the possibility that CCK is involved in the expression of panic symptoms is intuitively appealing. Indeed, a potential role for CCK in panic was initially discovered by Jens Rehfeld. Rehfeld (2000) reported that in 1979, he and a colleague were investigating whether CCK administration might trigger growth hormone secretion. To this end, they injected one another with a bolus of CCK-4. Unexpectedly, both individuals rapidly experienced what was described as a "fullblown attack including all of the classic symptoms of panic", which for lasted 20-25 minutes (Rehfeld, 2000, p. 80). de Montigny (1989) reported that intravenous CCK-4 (but not CCK-8s) administration in healthy volunteers induced significant anxiety or panic-like reactions in 10/10 participants. Subsequent studies with humans tended to focus on CCK-4 as opposed to CCK-8s. CCK-4 is the smallest fragment of the peptide that exhibits a high affinity for CCK-2 receptors. Because of its relatively small size, it has traditionally been assumed that CCK-4 is more likely to cross the blood brain barrier, compared to CCK-8s (de Montigny, 1984). The ability of CCK-4 to provoke panic attacks was subsequently demonstrated in panic disorder

patients, and reports from patients in these studies indicate that, from a phenomenological perspective, CCK-4-induced attacks were comparable to their naturally occurring bouts of panic (Bradwejn, Koszycki, & Materissian, 1990; Bradwejn, Koszycki, Payeur, Bourin, & Borthwick, 1992). Finally, CCK-4-induced panic is comparable (in terms of number and severity of symptoms) to the panic induced by the well-established panicogenic agent, carbon monoxide (Bradwejn & Koszycki, 1991)

In both healthy volunteers and individuals diagnosed with panic disorder, the panicogenic effects of CCK-4 are dose dependent. However, panic disorder patients demonstrate a higher sensitivity to the compound compared to healthy individuals, in terms of number of symptoms elicited, severity of symptoms, and duration of attack (Bradwejn, Koszycki, Annable, et al., 1992; Bradwejn, Koszycki, & Bourin, 1991). Amongst panic disorder patients, a linear relationship also exists between CCK-4 dose and increases in heart rate and diastolic blood pressure. It has been hypothesised that these CCK-induced physiological changes might arise from stimulation of neurons within brainstem regions such as the nucleus of the tractus solitarius, which contains both CCK-1 and CCK-2 receptors. Activation of CCK receptors within this, and additional brainstem structures (possibly the parabrachial nucleus and/or the medullary nuclei), might then lead to excitation and/or inhibition of afferent neurons projecting rostrally to higher order projection sites, giving rise to the affective and cognitive symptoms of panic (Bradwejn, 1993).

The panicogenic properties of CCK-4 are attenuated following pre-treatment with established anti-panic agents, including alprazolam (Zwanzger et al., 2003), lorazepam (de Montigny, 1989); imipramine, (Bradwejn, & Koszycki, 1994), citalopram (Shlik, Aluoja, Vasar, Podar, & Bradwejn, 1997), and fluvoxamine (van Megen, Westenberg, & den Boer, 1997), but not placebo. Acute pre-treatment with the selective CCK-2 receptor antagonists L-365,260 (Bradwejn et al., 1994), or to a lesser degree, CI-988 (Bradwejn et al., 1995) also attenuated the effects of CCK-4. However, negative results have also been reported when CI-988 has been used to block CCK-4-induced panic (van Megen, Westenberg, den Boer, et al., 1997), and L-365,260 failed to prevent naturally occurring panic attacks amongst individuals diagnosed with panic disorder (Kramer et al., 1995). The variable anti-panic effects associated with CCK-2 antagonists have been attributed to poor bioavailability related to these compounds (Bourin et al., 1998; Bradwejn & Koszycki, 2001).

1.2.3. Effects of CCK in Animal Models of Anxiety

When considering the effects of pharmacological compounds in purported "animal models of anxiety", it is important to recognise the limitations inherent in stating that behavioural changes in non-human animals represent a manifestation of "anxiety", per se (given that the experience of anxiety is a subjective, human experience). Typically, animal models are validated pharmacologically, whereby known anxiolytic compounds alter the behaviour in question in a consistent manner (e.g., increasing the amount of time a rat

explores an open, well-lit, novel environment), and known anxiogenic compounds alter the behaviour in the opposite manner (e.g., decreasing exploration time). This type of pharmacological validity is also referred to as "predictive validity" (Andreatini, Blanchard, Blanchard, Brandao, Carobrez, et al., 2001). In addition, researchers typically focus on species-specific behaviours that possess face validity as indicators of stress or anxiety. Often, the proposition that the behaviour under consideration represents a manifestation of stress or anxiety is based on the evolutionary significance of the behaviour for the species in question. For example, when rats explore open, well-lit, unfamiliar environments, the probability that it will be exposed to predation increases. As such, it is reasoned that engaging in this behaviour is likely stressful/anxiety-provoking for the animal. Numerous animal models of anxiety have been developed, based on these principles of predictive and/or face validity (see Griebel, 1995, for a review of different animal models of anxiety). The effects of CCK in animal models of anxiety will now be considered.

The literature assessing the effects of CCK in animal models of anxiety is vast, and comprehensive reviews of the relevant studies have been presented by Griebel (1999) and Rodgers & Johnson (1995). The majority of studies have focused on CCK-8s, CCK-8us, or CCK-4, and/or different CCK-1 and CCK-2 antagonists. These compounds have been assessed in terms of their ability to modify what is considered to represent anxiety-related behaviour, in many animal models including the elevated plus maze (Harro & Vasar, 1991), and

elevated zero maze (Matto, Harro, & Allikmets, 1997), light/dark box, novelty suppressed feeding (Rex et al., 1994), four hole box exploratory test (Daugé et al., 1989), antipredator defence (Pavlasevic, Bednar, Qureshi, & Södersten, 1993), acoustic startle (Frankland, Josselyn, Bradwejn, Vaccarino, & Yeomans, 1997), conditioned place preference (Valverde, Smadja, Roques, & Maldonado, 1997), punished lever pressing and Vogel punished drinking (Griebel, Perrault, & Sanger, 1997), open field exploration (Männistö et al., 1994), and periaqueductal grey (PAG) stimulation (Jenck, Martin, & Moreau, 1996).

Taken together, the results of studies focusing on both CCK agonists and antagonists are inconsistent. In this respect, Griebel (1999) reported that CCK-4 evoked an increase in anxiety-like behaviour in 48% of experiments, whereas CCK-8s was associated with an anxiogenic behavioural effect in 53% of studies. In the remaining studies, each of the compounds was without effect, or in a relatively few cases, anxiolytic. Use of CCK-8us yielded a similarly inconsistent behavioural profile. The picture was no clearer when the effects of CCK antagonists were considered. Specifically the CCK-1 receptor antagonist devazepide, and the CCK-2 receptor antagonist L-365,260 were associated with anxiolytic effects in only 50% of studies. While the CCK-2 antagonist CI-988 was found to be associated with the most consistent anxiolytic effects, negative results were reported in 26% of experiments. Finally, the anxiolytic effects associated with these two CCK-2 receptor antagonists tended to be relatively small (compared to benzodiazepine-associated anxiolysis) and dose-dependent

effects were generally lacking (Griebel, 1999). Several factors may be relevant when attempting to account for these discrepant findings, including inter-study methodological differences associated with the time of the year when testing was conducted, rat strain, housing and handling conditions, lighting during testing, and time during the light/dark cycle at which the testing was conduced (Fink et al., 1998; Griebel, 1999; Koks et al., 2000). The baseline anxiety level of subjects has been demonstrated to affect behavioural reactivity to CCK compounds. Subjects exhibiting relatively elevated baseline levels of anxiety typically have a stronger reaction to CCK-4 (Harro, Vasar, & Bradwejn, 1993).

The majority of animal models of anxiety were initially developed for, and validated with, traditional anxiolytics (i.e., benzodiazepines), and it has been suggested that some of these models might not be valid for screening the putative anxiolytic and anxiogenic effects of CCK compounds (Belzung, 2001; Griebel et al., 1997; Griebel, 1999). Given that CCK is proposed to be a key neurochemical in panic, it would be important that an appropriate animal model (i.e., one that models panic-like behaviour) be used in order to validly assess the effects of CCK agonist and antagonists (Rodgers & Johnson 1995). Indeed, Jenck et al. (1996) suggested that models based on conflict (e.g., punished responding), exploration (e.g., elevated plus maze), social interactions, and electrical stimulation of the PAG stimulation, may simulate different anxiety disorders (also see Bourin, 1997). If this is the case, then combining the results

across studies that have used different models may yield inaccurate data regarding the possible role of CCK in panic.

The PAG stimulation paradigm has been validated as a rat model of panic (Jenk, Martin, & Moreau, 1995). Peripheral administration of L-365,260 results in an antipanic effect in this model, although administration of CCK-4, butyloxycarbonyl-CCK-4 (BOC-CCK-4; a form of CCK-4 which is protected against enzymatic degradation), or CI-988 was without effect. The authors attributed the negative effects to poor central bioavailability of these compounds (Jenck et al., 1996). Griebel et al. (1997) tested CCK-1 and CCK-2 antagonists in two conflict models, two exploratory models, and in the mouse defensive test battery (MDTB), an ethologically based, putative model of panic. Only the CCK-2 antagonists exerted an anxiolytic effect, and this observation was limited to behavioural parameters as assessed in the MDTB.

Taken together, the CCK data from animal models of anxiety are inconsistent. Numerous inter-study methodological differences (even in studies using the same animal model of anxiety) make it difficult to determine whether CCK plays a significant role in the modulation of anxiety-like behaviour. That being said, there is some evidence to suggest that CCK antagonists might possess anxiolytic properties in models that are purported to be specific for panic-like behaviour.

There is both pharmacological and face validity to support the claim that isolation-induced UVs represent a manifestation of panic-related behaviour.

With respect to pharmacological validity, it will be recalled that a reduction in UVs has been demonstrated following pre-treatment with drugs that are used as anti-panic agents in the clinical setting. Turning to the issue of face validity, there are similarities between the behavioural features of human panic and isolation-induced UVs (e.g., the onset of behaviour is sudden and intense, and coupled with cardiorespiratory changes).

If isolation-induced UVs represent panic-like behaviour, it is important to consider the possible contribution of CCK as a neuochemical modulator of this behaviour. In this respect, there is converging evidence to suggest that CCK might be involved in modulating UVs. As noted above, CCK and CCK receptors are present early in the course of postnatal development. Both CCK and CCK receptors are localized in many brain regions that have been implicated in the production of UVs (including the brainstem, limbic system, and cerebral cortex). Of note, these central structures are also involved in the expression of anxiety in general, and panic in particular (see Zacharko et al., 1995 for a review). Based on these data, a thorough evaluation of the effects of CCK receptor agonists and antagonists on UVs is warranted.

2.0. EXPERIMENTS 1-4: EFFECTS OF BOC-CCK-4 AND CCK-8s ON ULTRASONIC VOCALIZATIONS, MOTOR ACTIVITY, AND BODY TEMPERATURE

2.1. Introduction: Experiments 1-4

Nine-day-old pups that are isolated as a group from the dam overnight exhibit an increase in plasma CCK once they are re-united with the dam (Weller et al., 1992). While the precise mechanisms underlying this effect are not clear, these findings indicate that levels of endogenous CCK in the rat pup can be affected by disturbing the contact between the pup and the dam. In this respect, studies that have directly assessed the effects of CCK agonists on isolation-induced UVs are limited. Weller and Blass (1988) reported that CCK-8s, but not CCK-8us, induced a significant reduction in UV rates in 11-day-old pups. These effects were present across a wide range of doses (1μg/kg - 20 μg/kg), and were specific in that indices of motor activity were not affected by CCK-8s, nor was paw lift latency from a hotplate (a measure of analgesia). Although changes in body temperature were not assessed in this study, the authors reported that at least some of the doses of CCK-8s that reduced UVs had no effect on body temperature (based on their unpublished data). CCK-8s had no effect on the ability of morphine to reduce UVs, and actually exerted somewhat of an additive effect, further decreasing UV rates in morphine-treated animals compared to control subjects. Naltrexone, an opioid antagonist, reliably blocked morphine's

effects, and this effect was in turn reversed by CCK-8s in both 3- and 11-day-old pups.

Taken together, these findings suggest that a functional (agonistic) interaction could exist between CCK and opioid systems in the developing pup. Weller and Blass (1988) suggested that the effects of CCK-8s might have been mediated by peripheral CCK-1 receptors (at least in 3-day-old subjects), given that there are few functional CCK receptors in the central nervous system at that age (they did not speculate regarding whether the effects of CCK-8s in 11-day-old pups were mediated peripherally and/or centrally).

While the findings by Weller & Blass (1988) suggest a specific role for exogenous CCK-8s modulation of isolation-induced UVs, a subsequent study carried out by the same group of researchers (Weller & Dubson, 1998) failed to detect any significant effects of CCK-8s on UV rates in 6- to 9-day-old pups. The authors point to significant methodological differences (e.g., age of pups at testing, duration of time away from the dam prior to testing, ambient temperature during testing) between the two studies, which might have accounted for these discrepant findings. In addition, low UV rates amongst control subjects might have precluded detection of CCK-8s anxiolysis, owing to a floor effect. The effects of CCK-8s on UVs were assessed in 5-day-old pups by Rex et al. (1994). In contrast to the findings of Weller & Blass (1988), but consistent with Weller and Dubson (1998), CCK-8s did not significantly affect UVs in this study (although UV rates were reduced by over 50% in the CCK-8s-

treated group, this effect was not statistically significant). However, pre-treatment with BOC-CCK-4 induced a marked (i.e., 1199%) increase in UVs, over a 5-minute test period. Measures of motor activity and body temperature were not conducted (Rex et al.).

A comparison of the results from the experiments conducted by Weller and Blass (1988), Weller and Dubson (1998), and Rex et al. (1994) indicated that CCK-8s exerted anxiolytic effects in 11-day-old pups but was without effect in 5- to 9-day-old pups. While CCK-8s reversed the effects of naltrexone in pups as young as 3 days old, the drug was not administered to 3-day-old pups on its own in the Weller and Blass (1988) study, and conclusions regarding its independent effects at that age can not be reached based on their data. In contrast to the effects of CCK-8s on UVs, BOC-CCK-4 has been shown to provoke a marked increase in UVs in 5-day-old pups. However, the data with respect to BOC-CCK-4 are limited to the results of a single study, and without concurrent measurements of motor activity, or body temperature, it is not possible to state whether the effects on UVs are behaviourally specific.

The current set of studies assessed the effects of BOC-CCK-4 and CCK-8s on UVs, motor activity, and body temperature in 12-day-old Long Evans hooded rat pups. Two different pre-treatment times were investigated in order to assess potential time-dependent effects. In this respect, in the Weller and Blass (1988) study, CCK-8s exerted anxiolytic effects 5 to 25 minutes following drug treatment. In contrast, Weller and Dubson (1998) and Rex et al. (1994) found

that CCK-8s had no significant effect on UVs when the fragment was administered 15 minutes prior to testing. In the current set of experiments, CCK-8s was administered 15 or 30 minutes prior to testing, in order to examine whether there were any time-dependent UV effects under conditions where other methodological variables were held constant. BOC-CCK-4 was also administered either 15 or 30 minutes prior to testing, also to assess for possible time-dependent effects, and in order to compare the effects of BOC-CCK-4 and CCK-8s within the current set of experiments. Based on the existing literature, it was hypothesized that CCK-8s would reduce UVs in a selective manner, given that CCK-8s has been shown to reduce UVs in older (i.e., 11-day-old), but not younger (i.e., 6- to 9-day-old) pups (see above). Given that there are no published data regarding the UV effects of BOC-CCK-4 on relatively older pups, no formal hypothesis was offered for this compound (although if developmental factors are ignored, one might expect an increase in UV rates, given the previously detailed findings in younger pups).

In the current set of experiments, 12-day-old pups were used for several reasons. First, as previously noted, baseline UV rates tend to be elevated during this period of development (thus allowing for the detection of potential drug-induced increases or reductions in UV rates). Second, as pups age, they are able to engage in different types of motor behaviour (versus younger pups) (Spear, 1990). This allows for the assessment of CCK fragment effects on multiple

indices of motor activity. Finally, at 12 days of age, CCK receptors are plentiful in the rat forebrain (Hays et al., 1981).

2.2. Method: Experiments 1-4

2.2.1. Subjects

Subjects used in all of the experiments were offspring of Long Evans hooded rats, purchased from Charles River Canada (St. Constant, Quebec), and bred in colony rooms in the Department of Psychology at Dalhousie University. Adult rats were initially housed for a ten-day mating period, in male-female pairs, or in cages consisting of one male and two females. The cages were constructed from Plexiglas (23 x 45 x 15 cm) with wire mesh tops and wood chips for bedding. The colony room was maintained at a temperature of 22± 1°C, and subjects were housed on a reversed 12-hour light-dark cycle (lights off at 09:30). Purina Rodent chow #5001 and water were available ad libitum.

Following the ten-day mating period, the male and female adult rats were separated and housed in individual cages. Each cage containing a mated female was checked for pups on a daily basis, starting 21 days after the first day of mating. When a litter of pups was born (Day 0), shredded paper was added to the cage as nesting material, and the cage was marked with a tag indicating that the bedding was not to be changed. On postnatal Day 5, each litter was culled to 10 pups, irrespective of sex, and the tag noting that the bedding was not to be changed was removed.

80 subjects were used in each experiment, with 16 subjects randomly assigned to each of five treatment conditions. Litters containing from 5 to 9 pups were also included, but the litter was not culled. Pups remained with the dam until the day of testing (Day 12). Behavioural testing was conducted during the subjects' dark cycle.

2.2.2. Drugs

In Experiments 1 and 2, the CCK-2 receptor agonist BOC-CCK-4, purchased from Sigma Pharmaceuticals (St. Louis, U.S.A.), was initially dissolved in two drops of dimethylsulfoxide (DMSO), and then distilled water. A DMSO/distilled water mixture served as the vehicle for the control group in both experiments. In Experiments 3 and 4, CCK-8s, purchased from Sigma Pharmaceuticals, (St. Louis, U.S.A.), and was dissolved in distilled water. Distilled water served as the vehicle for the control group in both experiments.

All injections were administered intraperitoneally (i.p.) in a 2-ml/kg volume, using a 100 μl syringe (Becton-Dickenson & Co., Franklin Lakes, N.J., U.S.A.).

2.2.3. Apparatus

Axillary body temperature was recorded with a surface temperature probe (model SST-1 copper-constantan thermocouple: Physitemp Instruments, Clifton, U.S.A.), which was attached to a digital thermometer (Digi-Sense Model 8528-20: Cole-Palmer Instruments, Chicago, U.S.A.).

UVs were recorded with a capacitance microphone suspended approximately 10 cm above the centre of an enclosed, Plexiglas test chamber (26 x 15 x 12 cm). The microphone was attached to a bat detector (Model S-25: Ultra Sound Advice, London, U.K.). Broadband output from the bat detector was relayed to a custom-built (based on that described by Harrison & Holman, 1978) four-channel digitizer and set at 28 kHz, 36 kHz, 44 kHz, and 52 kHz. The digitizer sampled output from the bat detector in 0.1-second bins, and sent the results via a terminal panel and interface card (Strawberry Tree) to a Macintosh 2CX computer. The number of ultrasounds was then recorded cumulatively, on a minute-by-minute basis, across the four frequencies corresponding to each of the digitizer channels.

Motor activity was divided into four behaviours, which were recorded on a check sheet, by an observer (who was not blind to group assignment) seated directly in front of the previously described test chamber. Three lines were drawn with a felt marker on the outside surface of the chamber floor (each line by separated by 6-cms.). The frequency of each of the four motor behaviours was recorded, and behaviours were summed over the duration of the entire test interval:

i) Line Crossings: A "Line Crossing" was recorded each time the full length of the subject (excluding the tail) crossed over one of lines on the floor of the chamber.

ii) Turns: A turn was recorded each time the subject rotated its body and moved both forepaws at least 90° to the left or right of an imaginary vertical axis running through the centre of the subject's body.

iii) Head Raise: A "Head Raise" was recorded when the subject tilted its head upward, approximately 45° above an imaginary horizontal plane, running from the subject's snout through to its tail. A single head raise was considered to have terminated once the subject had returned its head to its resting position, below the aforementioned horizontal line.

iv) Wall Climbing: "Wall climbing" was recorded when the subject placed both of its forepaws on the wall of the test chamber. A single bout of the behaviour was considered to have terminated once the subject replaced both its forepaws on the floor of the test chamber. Frequently, a head raise would progress to an instance of wall climbing, when the subject would initially tilt its head upward beyond the 45° cut-off associated with a head raise, and then place its forepaws on the test chamber wall. In such cases, a wall climb would "trump" a head raise, and only the former would be recorded.

2.2.4. Procedure

On the day of testing (Day 12) subjects were transported in their homecage with the dam, from the colony room to a room adjacent to the testing room, and left undisturbed for a 30-minute acclimatization period. Following the acclimatization period, a single subject was removed from the homecage and

transported to the testing room in a small container containing homecage bedding. The experimenter wore latex gloves at all times while handling the subjects in order to minimize heat and odour transfer. The subject was then weighed and its pre-injection body temperature was recorded.

A split-litter design was employed in Experiments 1-4, with 2 pups from each litter of 10 pups randomly assigned to each of the five treatment conditions in each experiment. For litters containing 5-9 pups, only five pups were used, with one pup randomly assigned to each of the 5 treatment conditions per experiment. Each pup was injected and tested only once.

In Experiments 1 and 2, each subject was injected with vehicle or one of four doses of BOC-CCK-4 (5.0 μg/kg, 10.0 μg/kg, 20.0 μg/kg, or 40.0 μg/kg). The injections were administered either 15 minutes (Experiment 1) or 30 minutes (Experiment 2) prior to the initiation of behavioural testing. In Experiments 3 and 4, each subject was administered vehicle or one of four doses of CCK-8s (0.5 μg/kg, 1.0 μg/kg, 2.0 μg/kg, or 4.0 μg/kg), either 15 minutes (Experiment 3) or 30 minutes (Experiment 4) before testing. Doses of BOC-CCK-4 and CCK-8s were selected based on the findings of Weller and Blass (1988) and Rex et al. (1994).

Following drug pre-treatment, the subject was placed in a holding cage containing homecage bedding. The holding cage was partially submerged in a warm water bath maintained at 35±1°C, in order to simulate the ambient temperature of the nest. Pups were not replaced in the homecage with the dam

and littermates following drug treatment in order to avoid the possibility that injected pups might alter the dam's behaviour towards it, and the untreated pups. Rex et al. (1994) employed a similar procedure, whereby subjects were removed from their mother immediately before testing, treated with vehicle, BOC-CCK-4, or CCK-8s, and then placed alone in the experimental room (maintained at 37°C) until the initiation of behavioural testing. In contrast, in other studies that have examined the UV effects of CCK fragments (e.g., Weller and Blass, 1988) or a CCK-1 antagonist (e.g., Weller & Gispan, 2000), subjects were treated with vehicle or the test compound, and then returned to the homecage, which contained the remaining litter members and the anesthetized dam. It is clear that the manner in which pups are maintained during the post-treatment interval prior to testing represents yet another source of inter-study variability that makes direct cross-study comparisons difficult.

Turning back to the current set of experiments, after treatment with vehicle or a CCK fragment, each pup was subsequently removed from the holding cage either 15 minutes (Experiments 1 and 3) or 30 minutes (Experiment 2 and 4) following drug pre-treatment, transported to the testing room, and placed in the centre of the testing chamber. Rates of UVs, line crossing, turning, wall climbing, head raises and wall climbing were recorded over a 6-minute period. The ambient temperature of the testing room was maintained at 22 ±1°C.

Immediately following the completion of behavioural testing, the post-test body temperature was recorded, and each subject was then placed in a cage separate from the dam and the untested littermates. This procedure was conducted so as not to disrupt the dam or the untested littermates.

2.2.5. Data Screening and Statistical Analyses

All statistical analyses were conducted using StatView 5.0 (1998). Prior to conducting any statistical analyses, several screening procedures were employed in order to reduce the variability in the data, and to ascertain the degree to which the data met the assumptions of normality and homogeneity of variance.

First, data for each of the experiments were inspected for outliers. Specifically, a data point was characterized as an outlier if it was equal or greater than two standard deviations from the treatment group mean. A breakdown of the number of outliers in each experiment, for each of the dependent variables is listed in Table 1. Once outliers were identified, each of these cases was altered, and assigned a raw score falling one number larger than the next most extreme score in the distribution (Tabachnick & Fidell, 2001). This method for dealing with outliers was selected rather than deleting cases in order to maximize sample sizes, and so that the equal distribution of littermates could be maintained across treatment conditions.

Following modification of each of the outlier scores, data were then analyzed to see if the distributions were normally distributed. First, data within each treatment group for each dependent variable in Experiments 1-4 were

Table 1: Breakdown of outliers in Experiments 1-4, across each of the dependent variables. Values within each cell of the tables represent one case expressed as the number of SDs away the subject's score fell from the treatment group mean.

Experiment 1: CCK-4 (15 Minute Pre-treatment)

	UVs	Line Crossings	Turns	Head Raises	Wall Climbing	Body Temperature Change
Vehicle	-	3.226	2.409	-	2.045 2.045	-2.120
5.0 µg/kg	-	2.582	-2.175	2.541	2.682 2.155	-
10.0 µg/kg	2.328	-	2.322	-	2.133	-2.064
20.0 µg/kg	-	3.019	-	2.041	2.113	-2.078
40.0 µg/kg	-	3.290	2.948	2.512	3.048	2.040

Experiment 2: CCK-4 (30 Minute Pre-treatment)

	UVs	Line Crossings	Turns	Head Raises	Wall Climbing	Body Temperature Change
Vehicle	-	2.538	2.249	-	3.056	-2.740
5.0 µg/kg	-	3.530	-	-	2.375	-2.035 2.769
10.0 µg/kg	2.328	3.214	-	-	2.801	-2.292 2.193
20.0 µg/kg	-	2.972	2.190	-	3.480	-
40.0 µg/kg	-	2.882	2.422	-	2.589	2.219

Table 1: (continued)

Experiment 3: CCK-8s (15 Minute Pre-treatment)

	UVs	Line Crossings	Turns	Head Raises	Wall Climbing	Body Temperature Change
Vehicle	2.331	3.152	-	2.406	3.307	-
0.5 μg/kg	2.224	3.490	-	-	2.780	2.765
1.0 μg/kg	-	3.130	2.067	2.149	2.128	-2.060 2.012
2.0 μg/kg	2.798	3.432	2.060	-	2.429	-2.002
4.0 μg/kg	2.102	3.338	-	-	3.343	-

Experiment 4: CCK-8s (30 Minute Pre-treatment)

	UVs	Line Crossings	Turns	Head Raises	Wall Climbing	Body Temperature Change
Vehicle	-	2.308 2.569	2.505 2.184	-	-	-2.054
0.5 μg/kg	2.054	-	2.275	-	2.160	-2.323
1.0 μg/kg	-	2.968	2.262	-	2.687	-2.319
2.0 μg/kg	-	2.580	2.526	2.028	-	-
4.0 μg/kg	-	3.288	2.931	2.061	-	-

tested by using the Kolmogorov-Smirnov test of normality. Data were then tested for homogeneity of variance between each of treatment groups across each of the dependent variables in Experiments 1-4, using Bartlett's Test of Homogeneity of Variance.

After data were tested for normality and homogeneity of variance, separate one-way analyses of variance (ANOVA) were conducted on rates of UVs, Line Crossing, Turning, Rearing, and Wall Climbing. In each of Experiments 1-4, Drug Dose (5 levels; vehicle and 4 doses of BOC-CCK-4 or CCK-8s) served as the between-group factor.

In order to calculate changes in body temperature associated with the respective drug treatment conditions, the pre-injection body temperature was subtracted from the post-test body temperature for each subject. Differences in the resulting variable, termed Body Temperature Change, were analyzed using a one-way ANOVA, with Drug Dose as the between-group factor.

Significant main effects associated with the between-group factor Drug Dose were subsequently analyzed with Fischer's PLSD post-hoc comparison technique.

2.3. Results: Experiments 1-4

2.3.1. Experiment 1: BOC-CCK-4 (15 Minute Pre-treatment) Modulation of UVs, Motor Activity, and Body Temperature

2.3.1.1. Ultrasonic Vocalizations

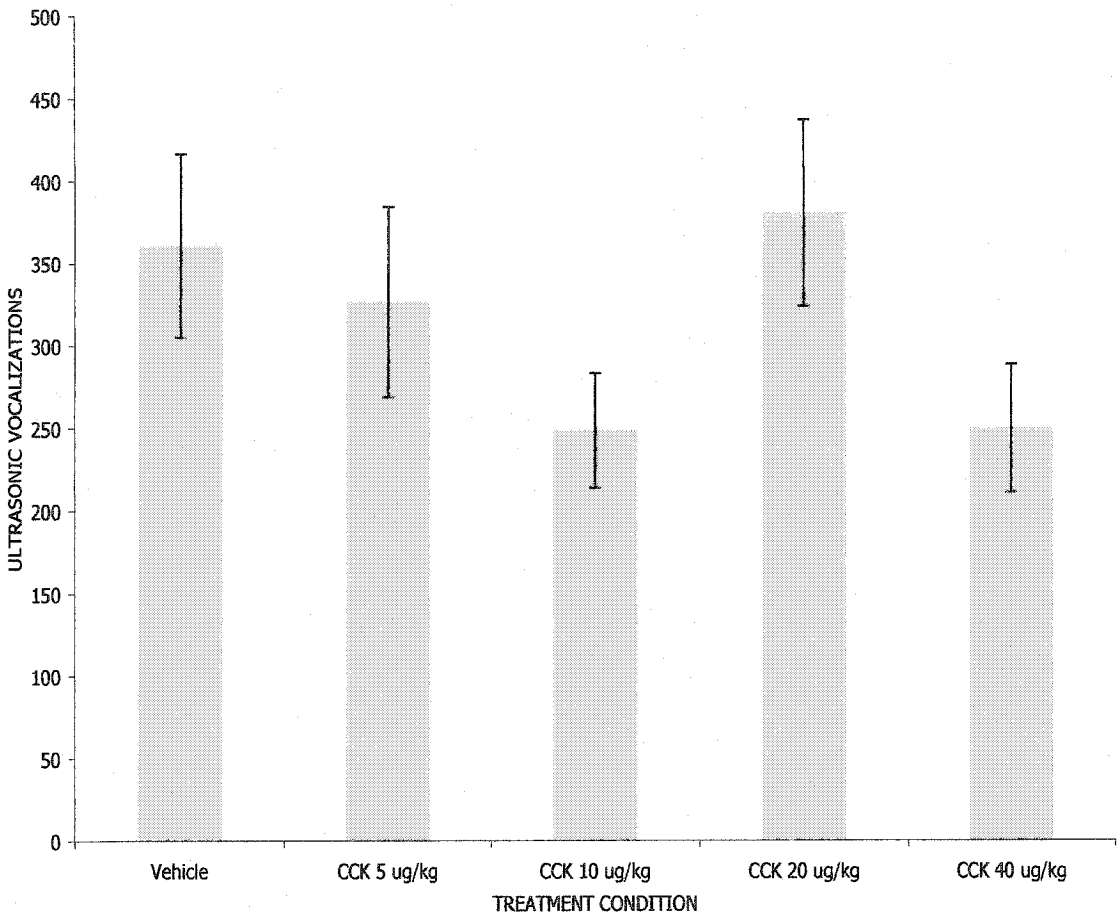
BOC-CCK-4 did not exert a statistically significant effect on UV rates ($F_{4,75}$ = 1.534, p = .2009; Figure 3a).

2.3.1.2. Motor Activity

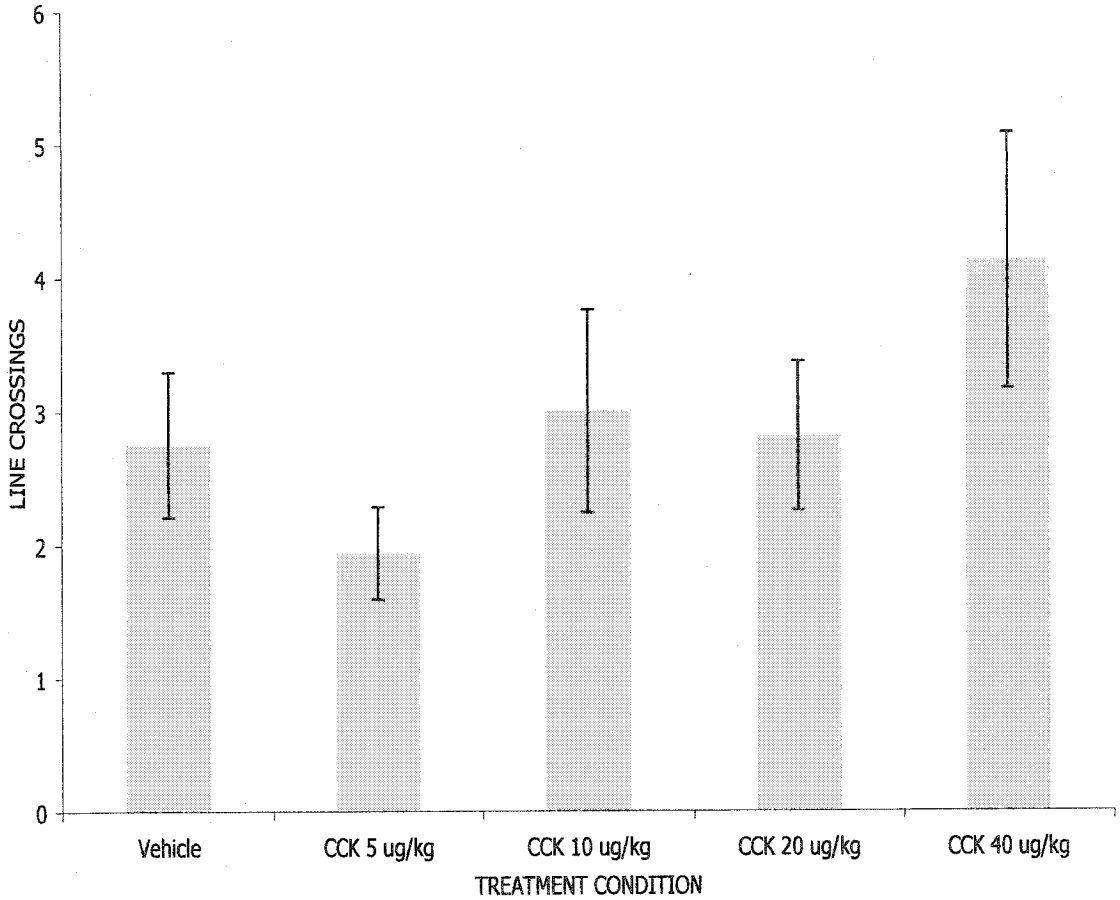
Separate ANOVAs conducted for each of the dependent variables associated with motor activity failed to detect a statistically significant main effect for drug dose when BOC-CCK-4 was administered 15 minutes prior to the initiation of behavioural testing. Specifically, BOC-CCK-4 did not exert a significant effect on Line Crossings ($F_{4,75}$ = 1.389, p = .2459; Figure 3b), Turns ($F_{4,75}$ = 1.229, p = .3058; Figure 3c), Head Raises ($F_{4,75}$ = 2.221, p = .0747; Figure 3d), or Wall Climbing ($F_{4,75}$ = 1.716, p = .1554; Figure 3e).

2.3.1.3. Body Temperature Change

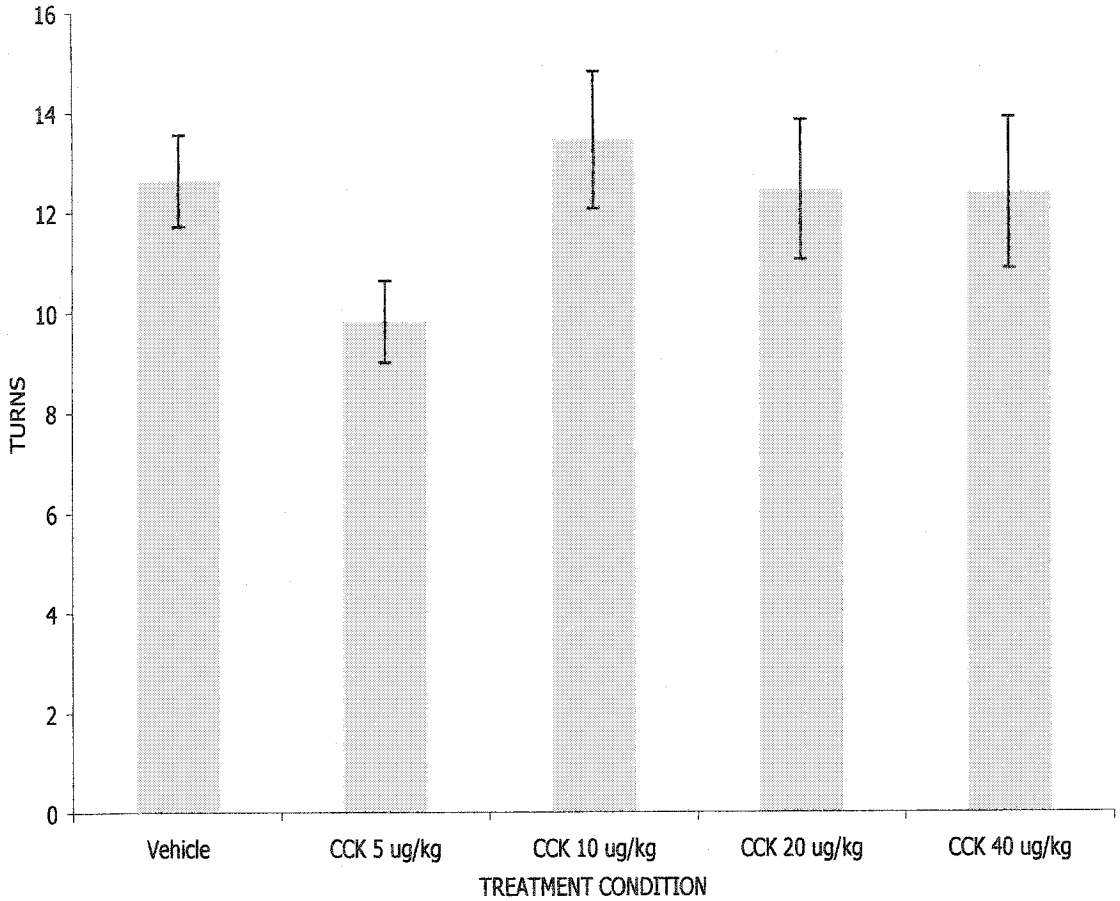
The ANOVA assessing the effect of BOC-CCK-4 on body temperature did not yield a significant effect ($F_{4,75}$ = 1.126, p = .3506; Figure 3f).



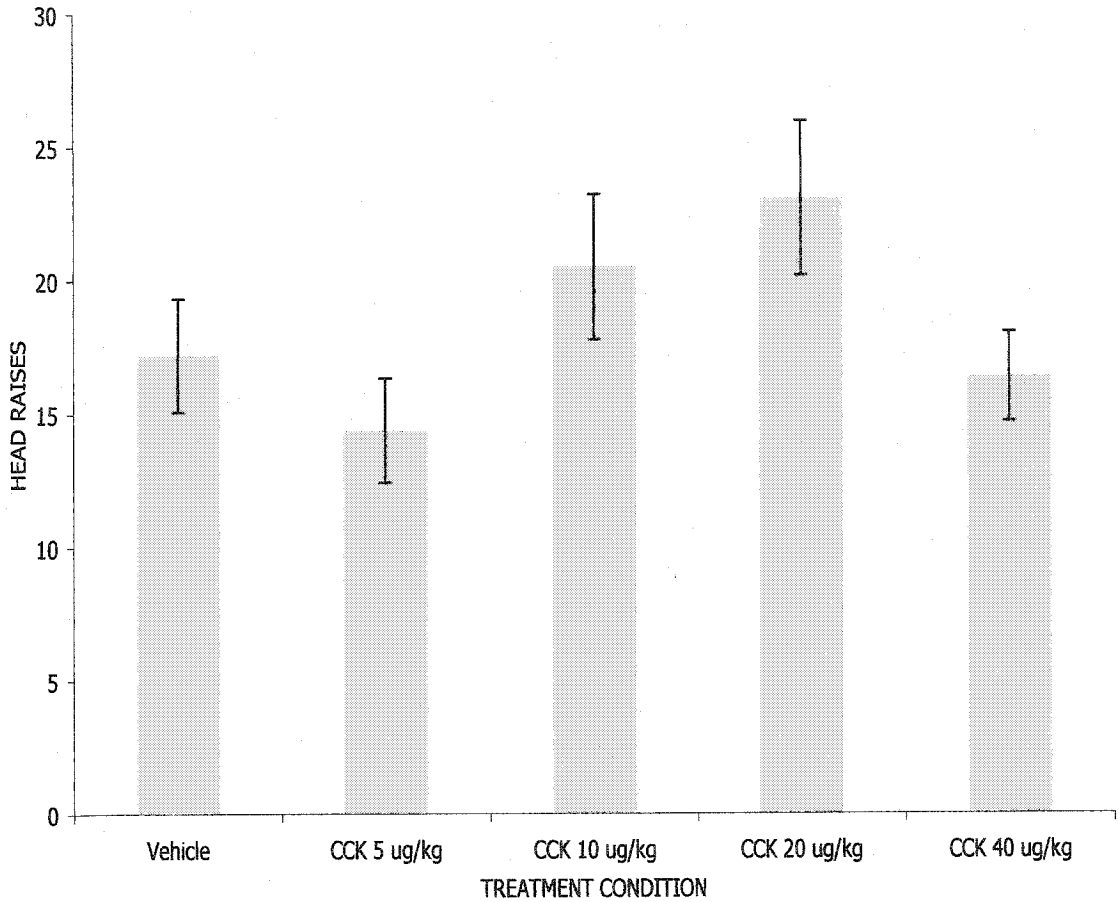
Figures 3a: Mean (±S.E.M.) UV rates over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 15 minutes following drug pre-treatment.



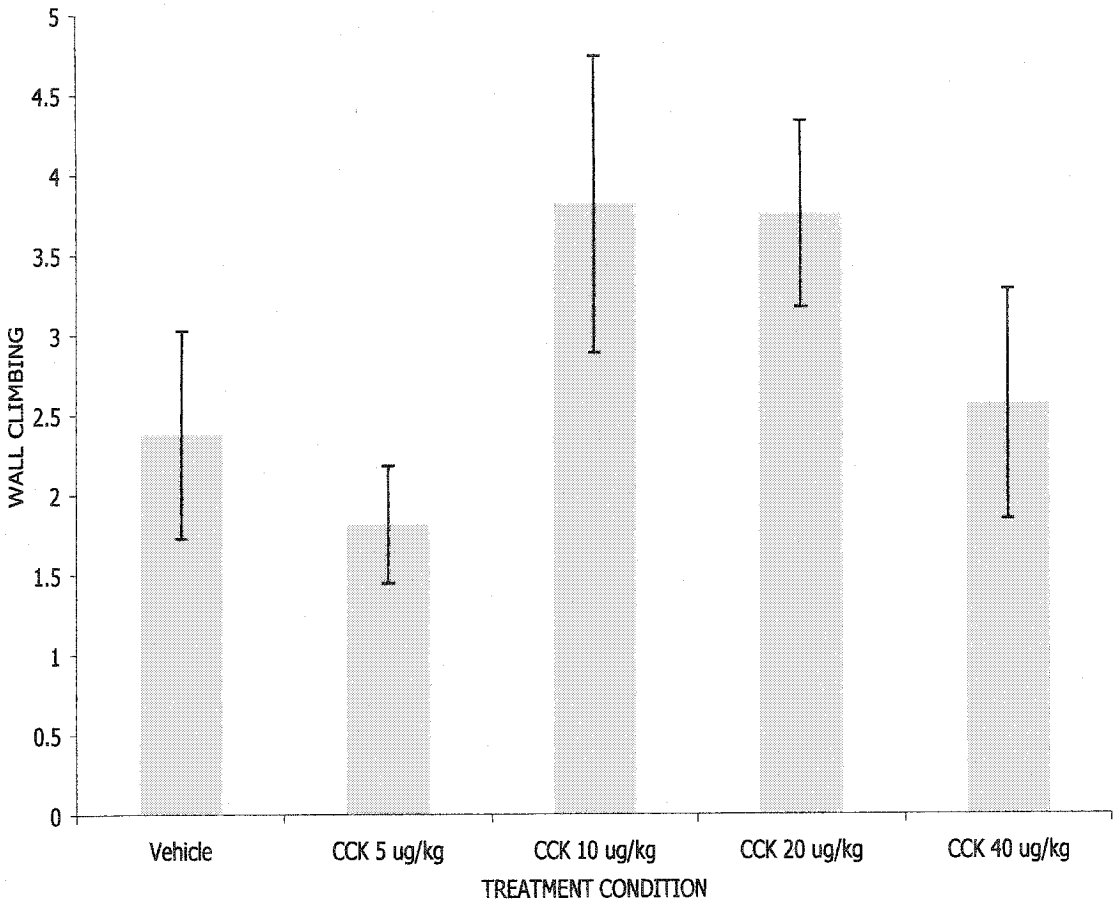
Figures 3b: Mean (±S.E.M.) Line Crossing rates over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 15 minutes following drug pre-treatment.



Figures 3c: Mean (±S.E.M.) rate of Turns over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 15 minutes following drug pre-treatment.



Figures 3d: Mean (±S.E.M.) rates of Head Raises over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 15 minutes following drug pre-treatment.



Figures 3e: Mean (±S.E.M.) Wall Climbing rates over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 15 minutes following drug pre-treatment.

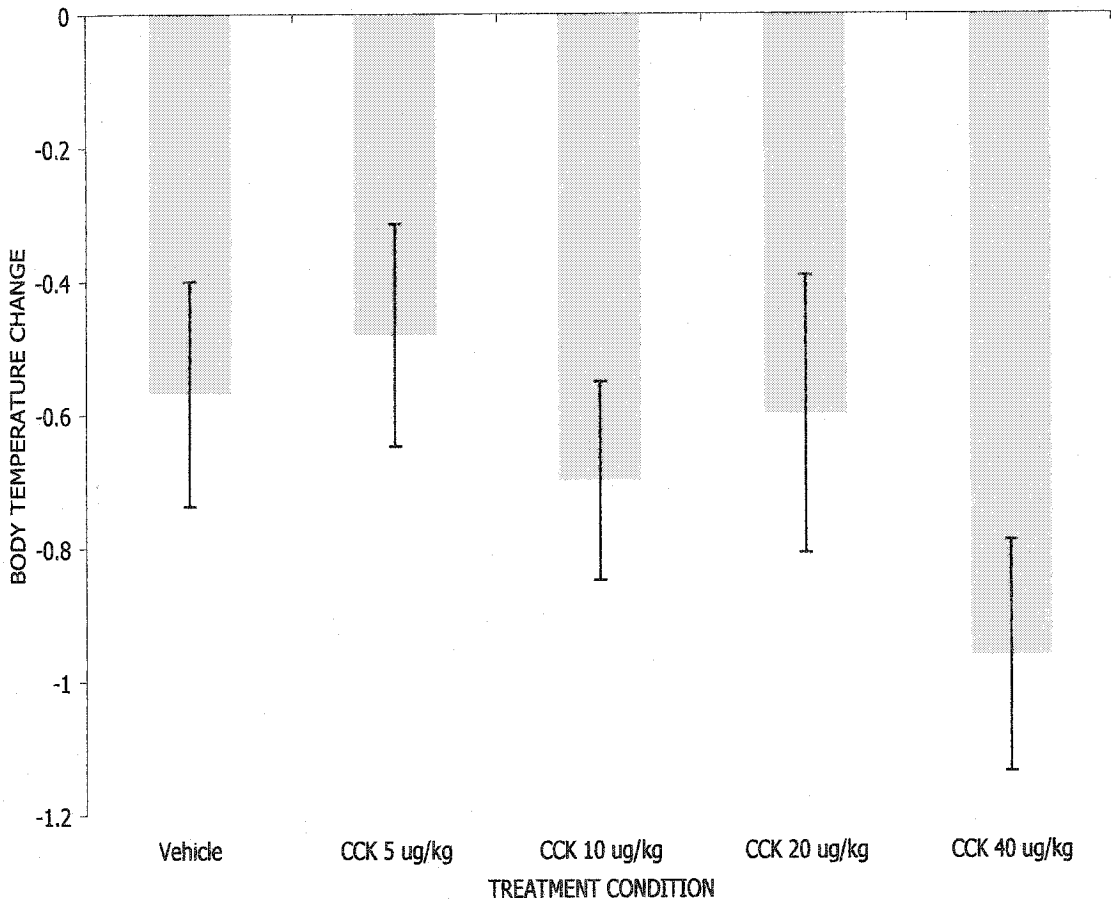


Figure 3f: Mean (±S.E.M.) Body Temperature Change following pre-treatment with vehicle or BOC-CCK-4, administered 15 minutes prior to behavioural testing.

2.3.2. Experiment 2: BOC-CCK-4 (30 Minute Pre-treatment) Modulation of UVs, Motor Activity, and Body Temperature

2.3.2.1. Ultrasonic Vocalizations

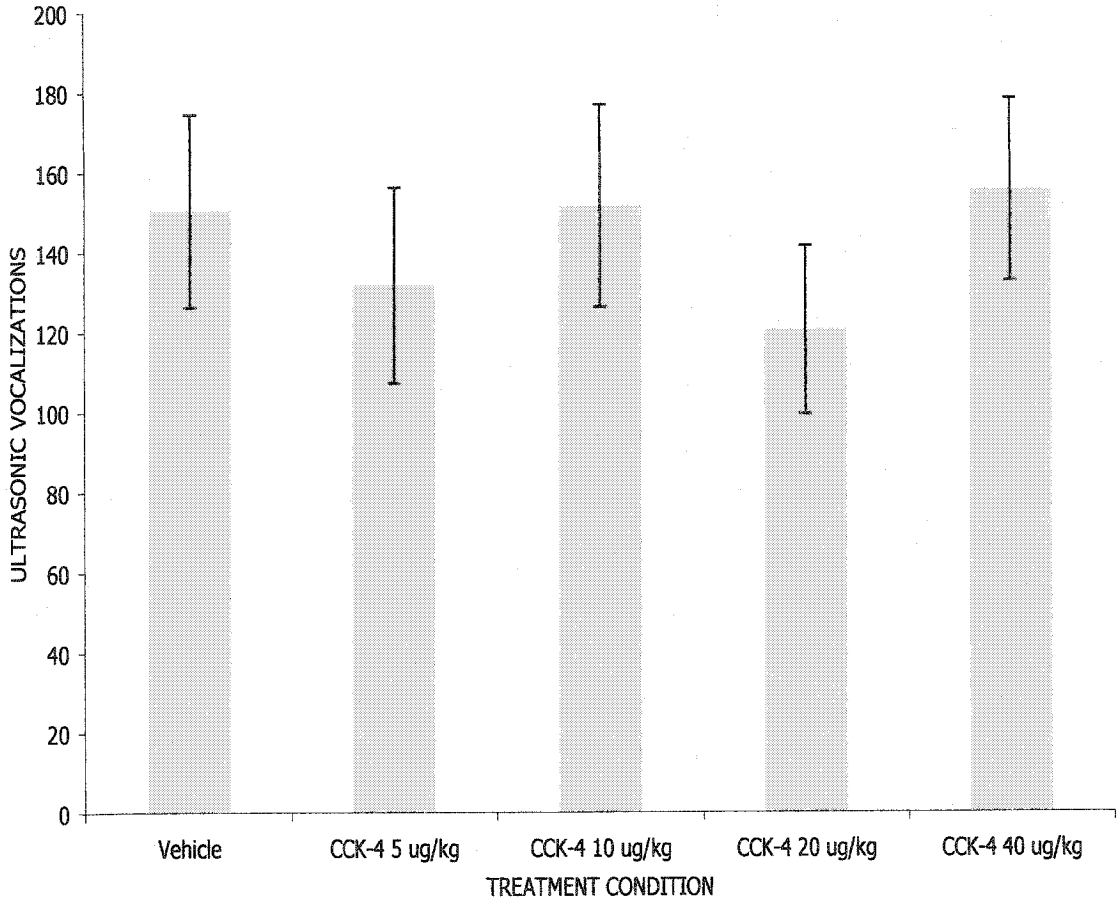
When administered 30 minutes prior to behavioural testing, BOC-CCK-4 did not alter ultrasonic rates to a significant degree ($F_{4,75}$ = .409, p = .8016; Figure 4a).

2.3.2.2. Motor Activity

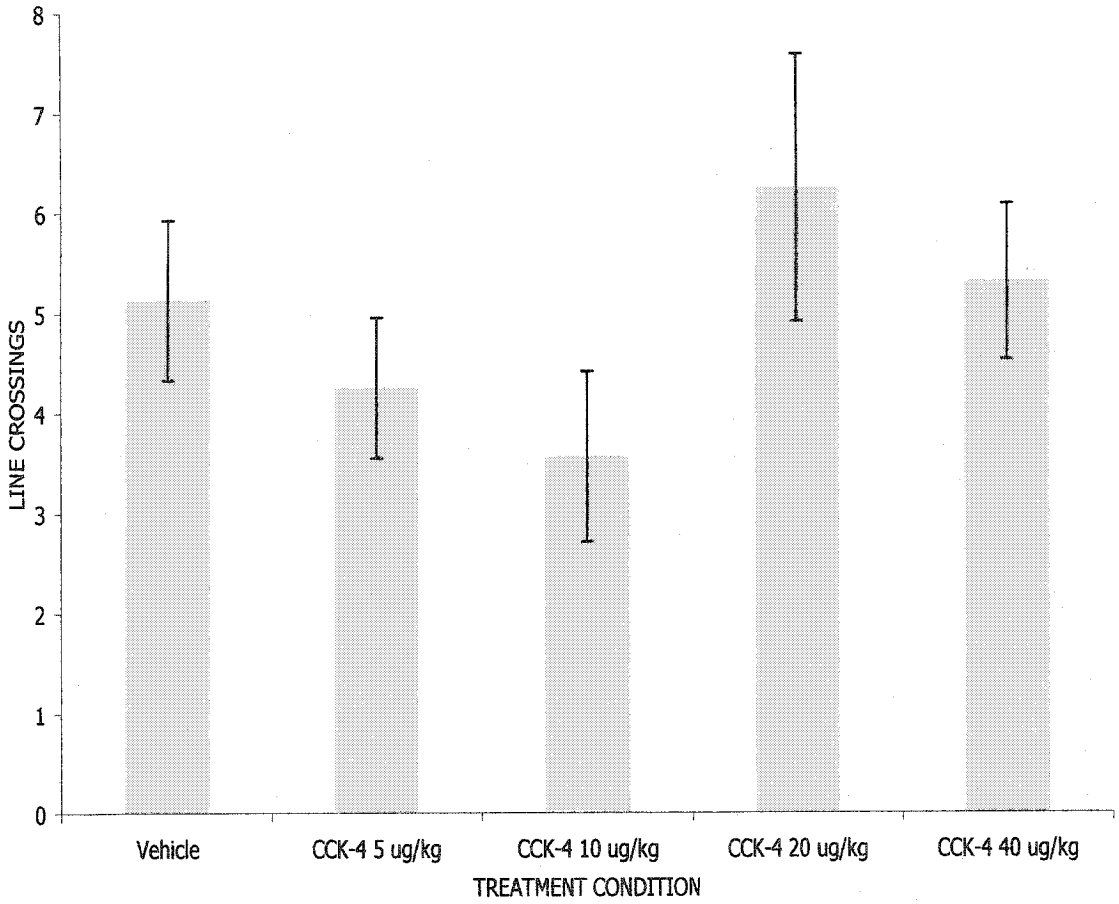
BOC-CCK-4 failed to exert a significant influence on any of the components of motor activity. Specifically, the compound did not alter the rates of Line Crossings ($F_{4,75}$ = 1.250, p = .2973; Figure 4b), Turns ($F_{4.75}$ = .402, p = .8067; Figure 4c), Head Raises ($F_{4.75}$ = 1.544, p = .1982; Figure 4d), or Wall Climbing ($F_{4,75}$ = .183, p = .9464; Figure 4e).

2.3.2.3. Body Temperature Change

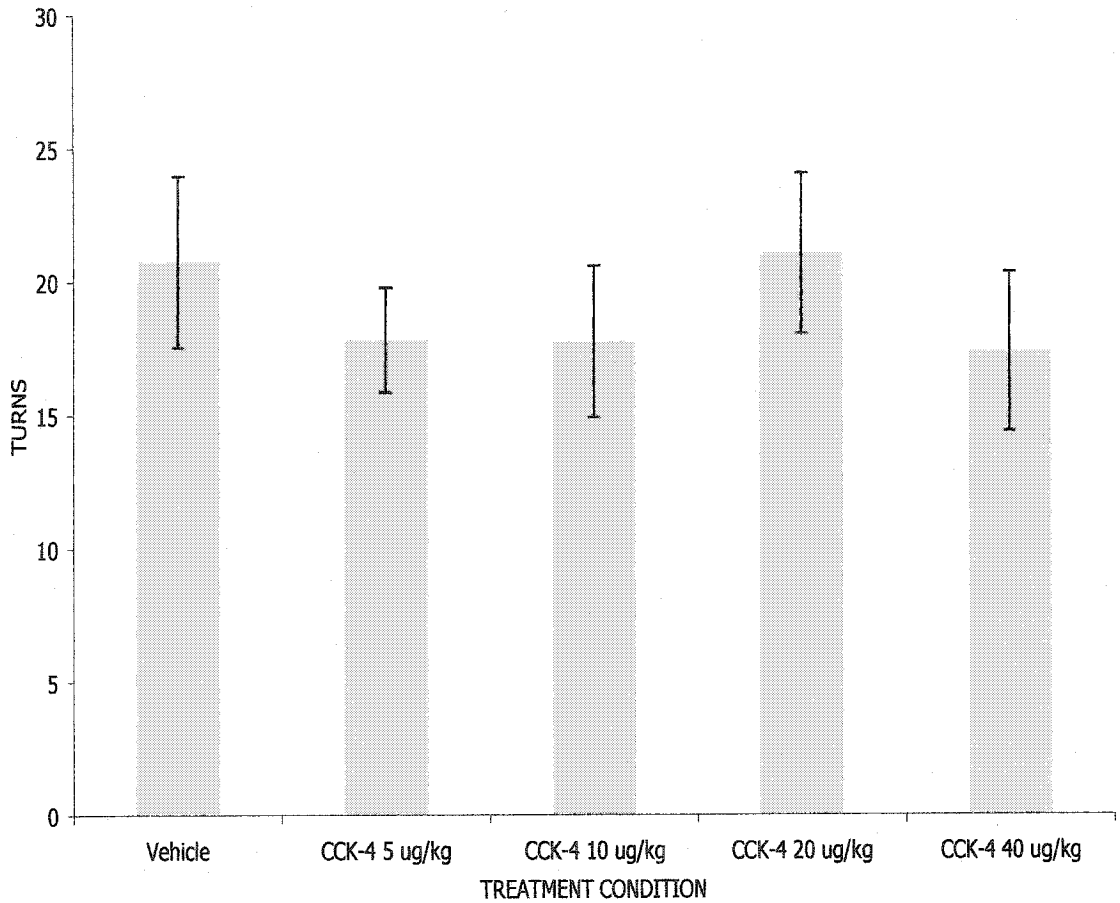
Consistent with the results of Experiment 1, Body Temperature was not significantly altered by BOC-CCK-4 when the compound was injected 30 minutes before testing ($F_{4.75}$ = 1.508, p = .2085; Figure 4f).



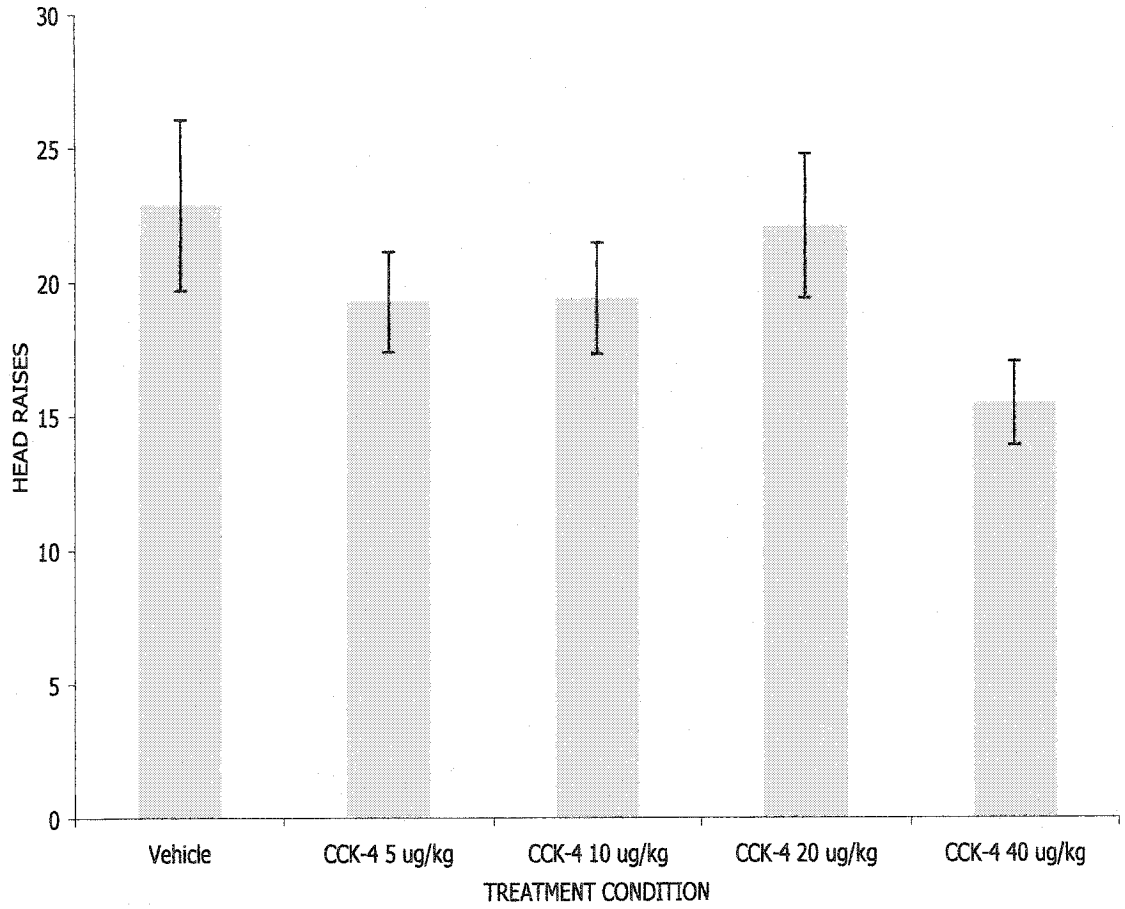
Figures 4a: Mean (±S.E.M.) UV rates over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 30 minutes following drug pre-treatment.



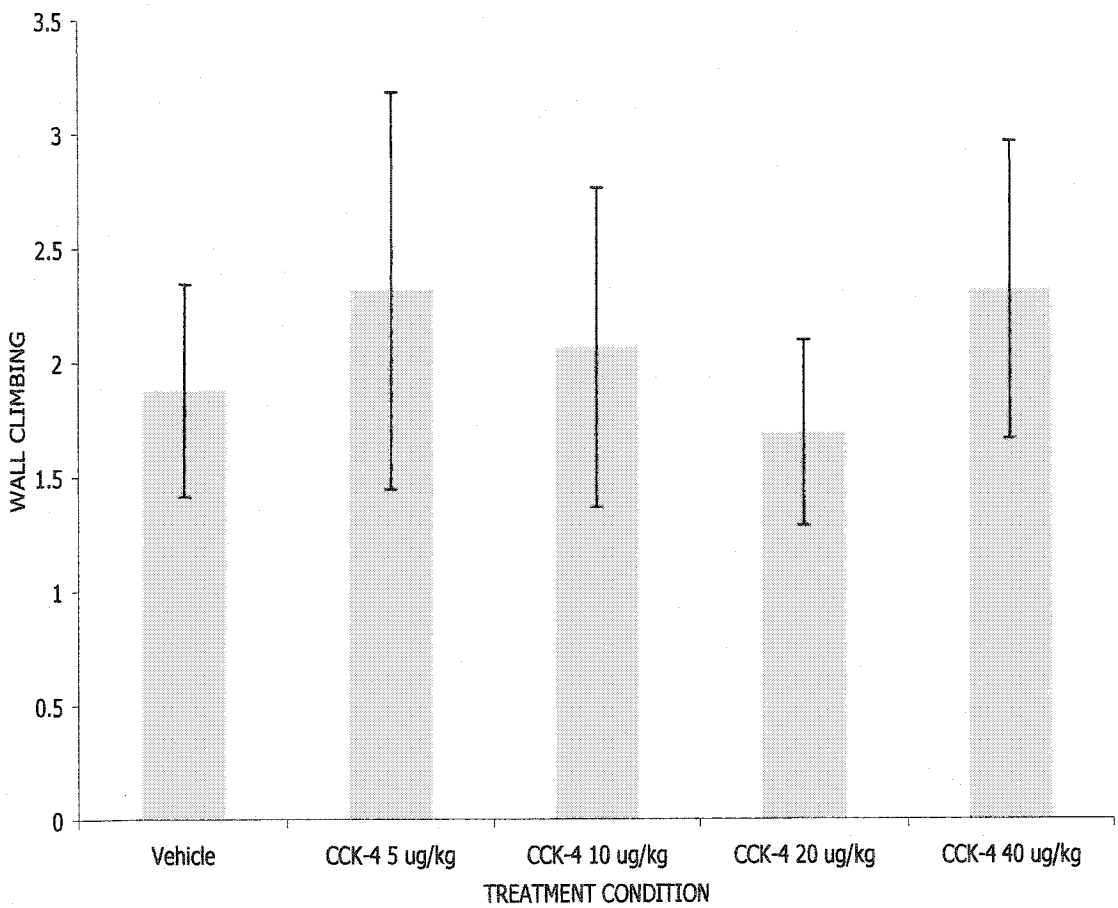
Figures 4b: Mean (±S.E.M.) Line Crossing rates over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 30 minutes following drug pre-treatment.



Figures 4c: Mean (±S.E.M.) rates of Turns over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 30 minutes following drug pre-treatment.



Figures 4d: Mean (±S.E.M.) rates of Head Raises over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 30 minutes following drug pre-treatment.



Figures 4e: Mean (±S.E.M.) Wall Climbing rates over a 6-minute period, following treatment with vehicle or BOC-CCK-4. Behavioural testing was conducted 30 minutes following drug pre-treatment.

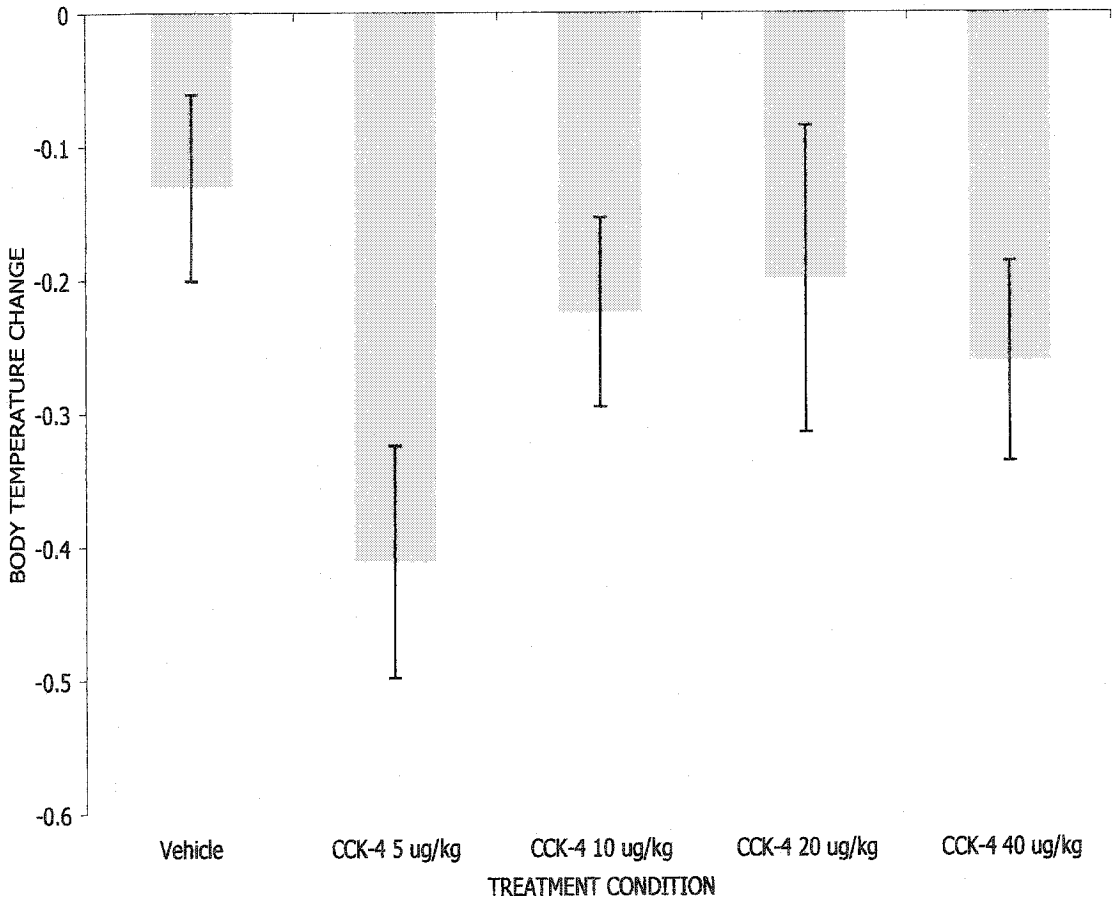


Figure 4f: Mean (±S.E.M.) Body Temperature Change following pre-treatment with vehicle or BOC-CCK-4, administered 30 minutes prior to behavioural testing.

2.3.3. Experiment 3: CCK-8s (15 Minute Pre-treatment) Modulation of UVs, Motor Activity, and Body Temperature

2.3.3.1. Ultrasonic Vocalizations

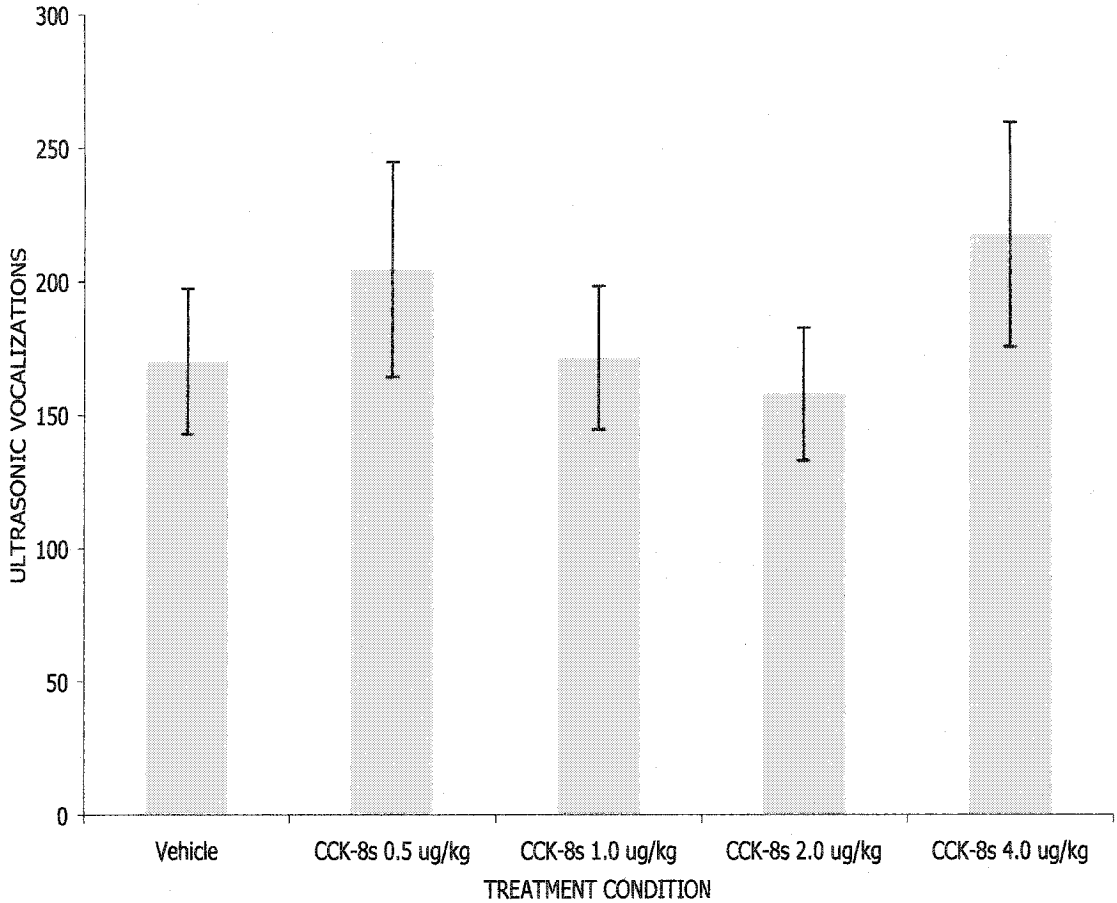
Following a 15-minute pre-treatment period, CCK-8s failed to significantly alter UV rates ($F_{4.75}$ = .593, p = .6688; Figure 5a).

2.3.3.2. Motor Activity

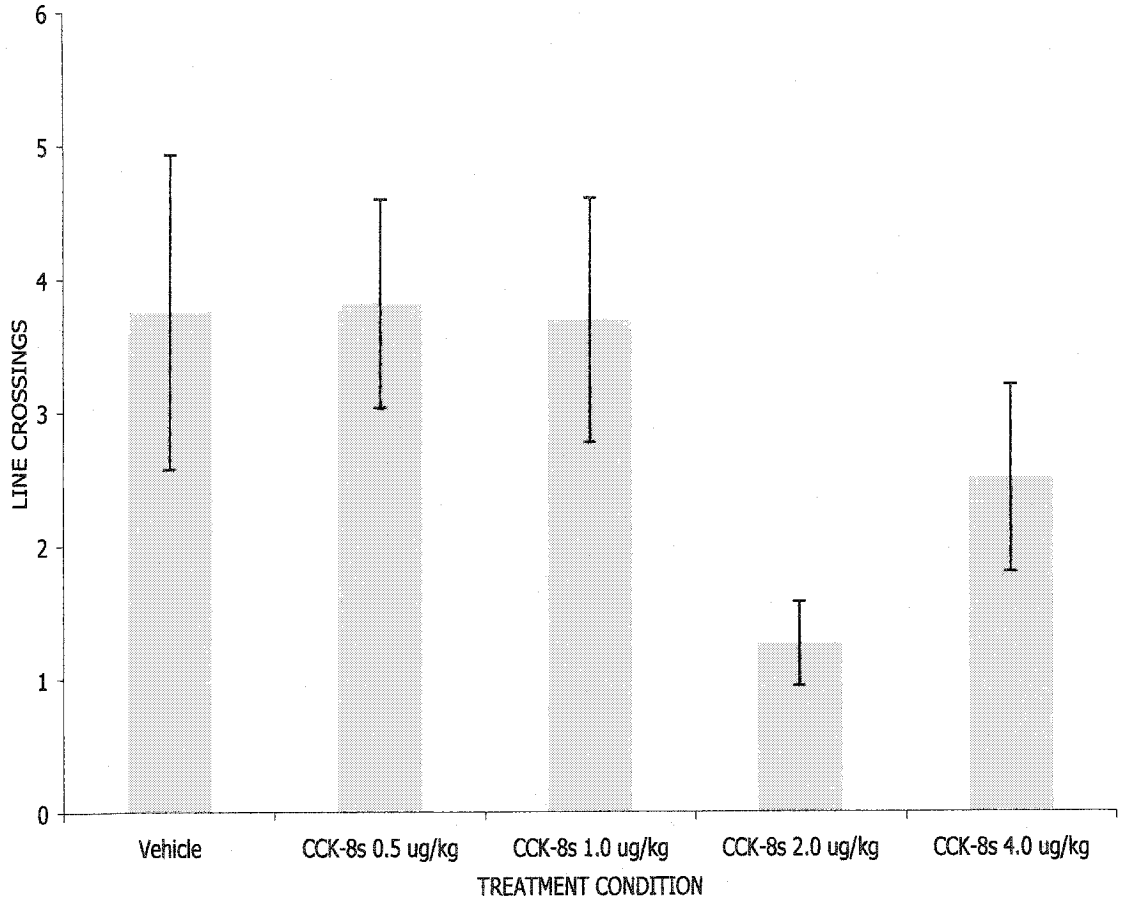
CCK-8s also failed to affect any of the measured indices of motor behaviour. In this respect, separate ANOVAs did not yield significant drug effects on Line Crossings ($F_{4.75}$ = 1.986, p = .1053; Figure 5b), Turns ($F_{4.75}$ = .704, p = .5918; Figure 5c), Head Raises ($F_{4.75}$ = .278, p = .8911; Figure 5d), or Wall Climbing ($F_{4.75}$ = .342, p = .8491; Figure 5e).

2.3.3.3. Body Temperature Change

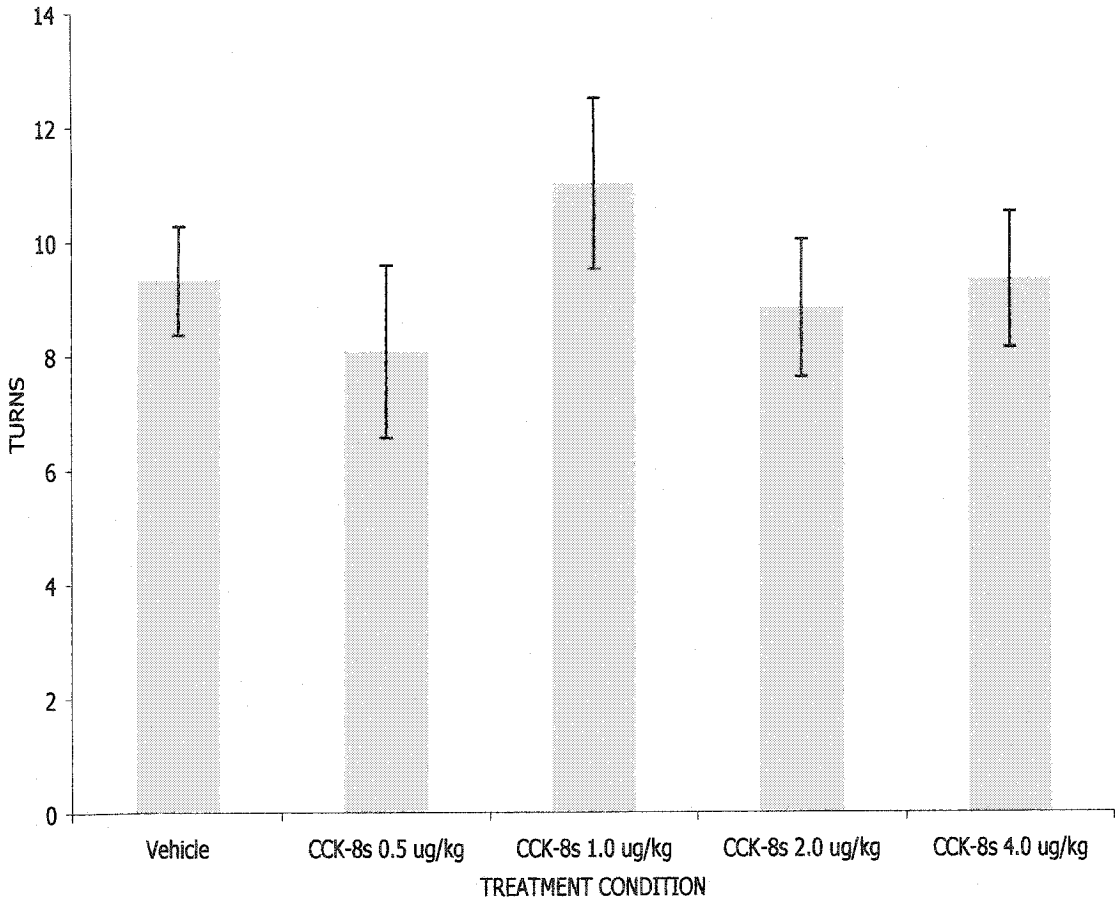
The ANOVA assessing the effect of CCK-8s on body temperature failed to detect a significant main effect ($F_{4.75}$ = 1.039, p = .3927) at any of doses tested (Figure 5f).



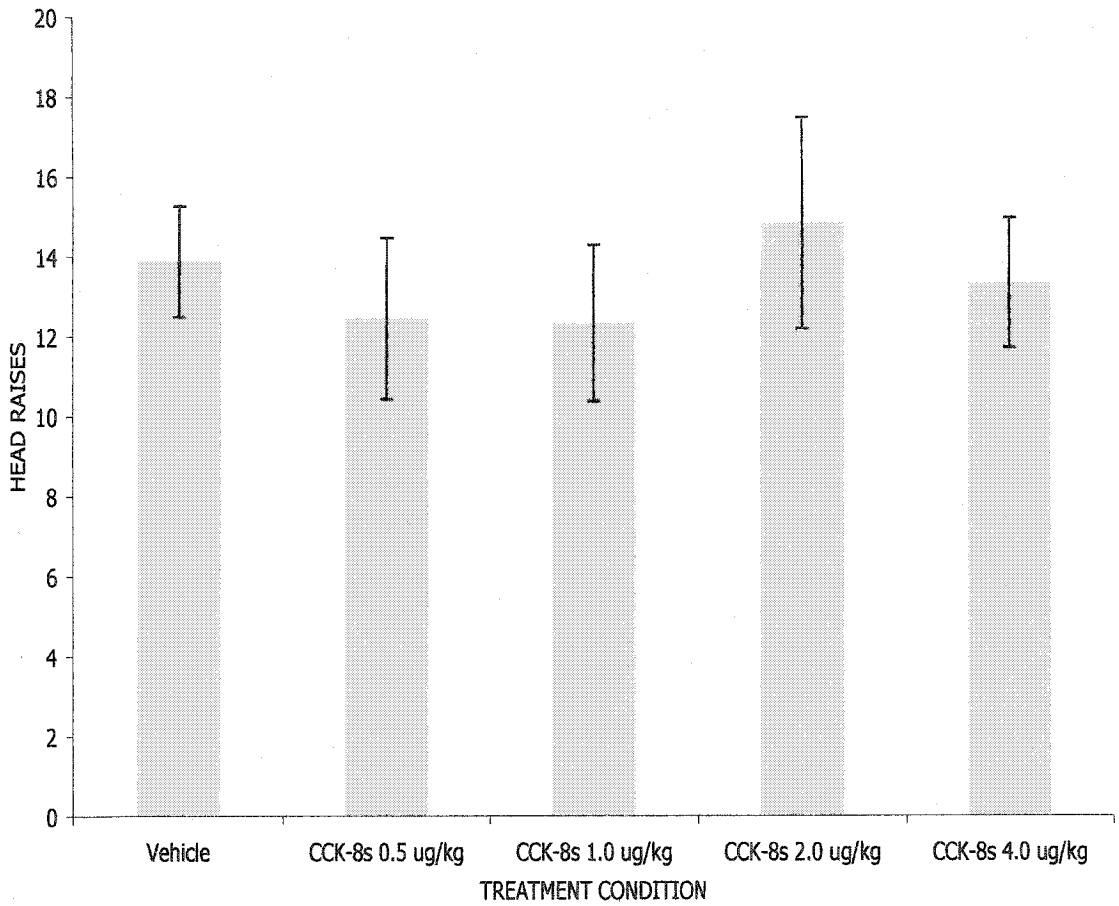
Figures 5a: Mean (±S.E.M.) UV rates over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 15 minutes following drug pre-treatment.



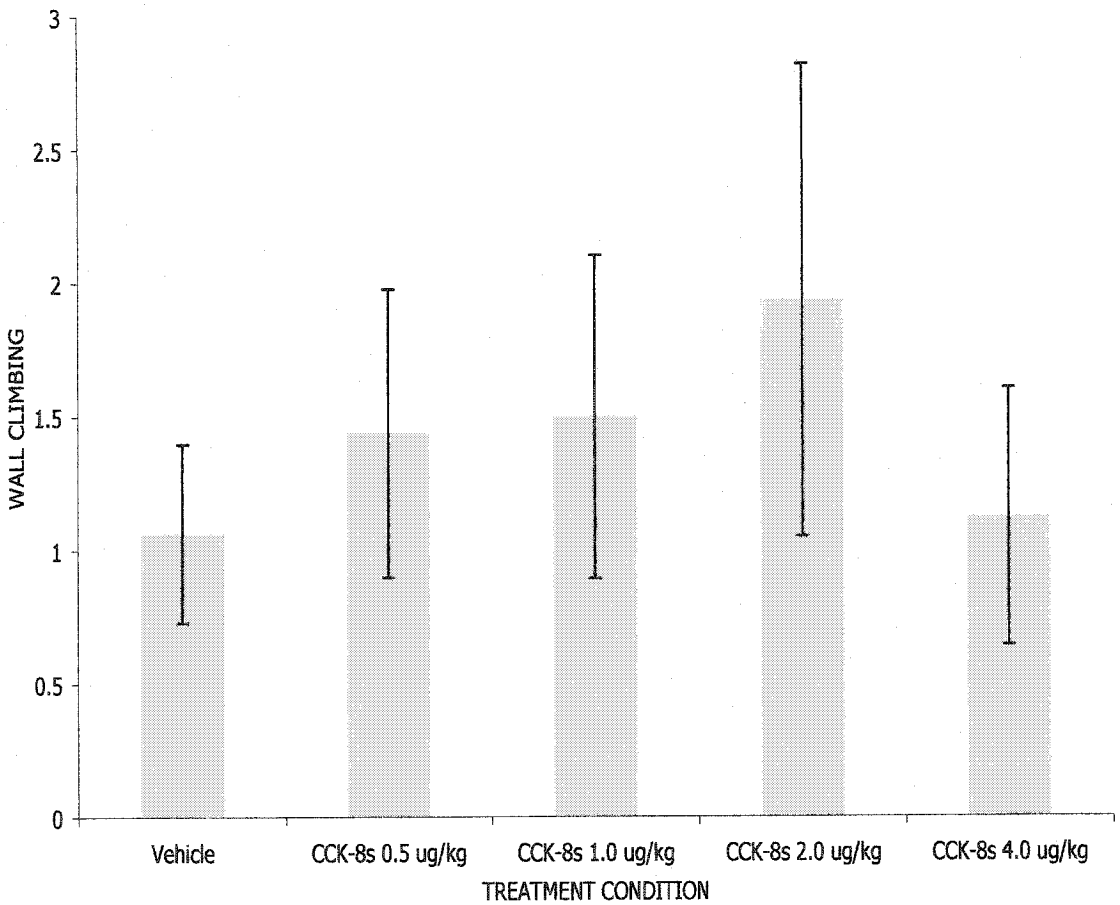
Figures 5b: Mean (±S.E.M.) Line Crossing rates over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 15 minutes following drug pre-treatment.



Figures 5c: Mean (±S.E.M.) rates of Turns over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 15 minutes following drug pre-treatment.



Figures 5d: Mean (±S.E.M.) rates of Head Raises over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 15 minutes following drug pre-treatment.



Figures 5e: Mean (±S.E.M.) Wall Climbing rates over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 15 minutes following drug pre-treatment.

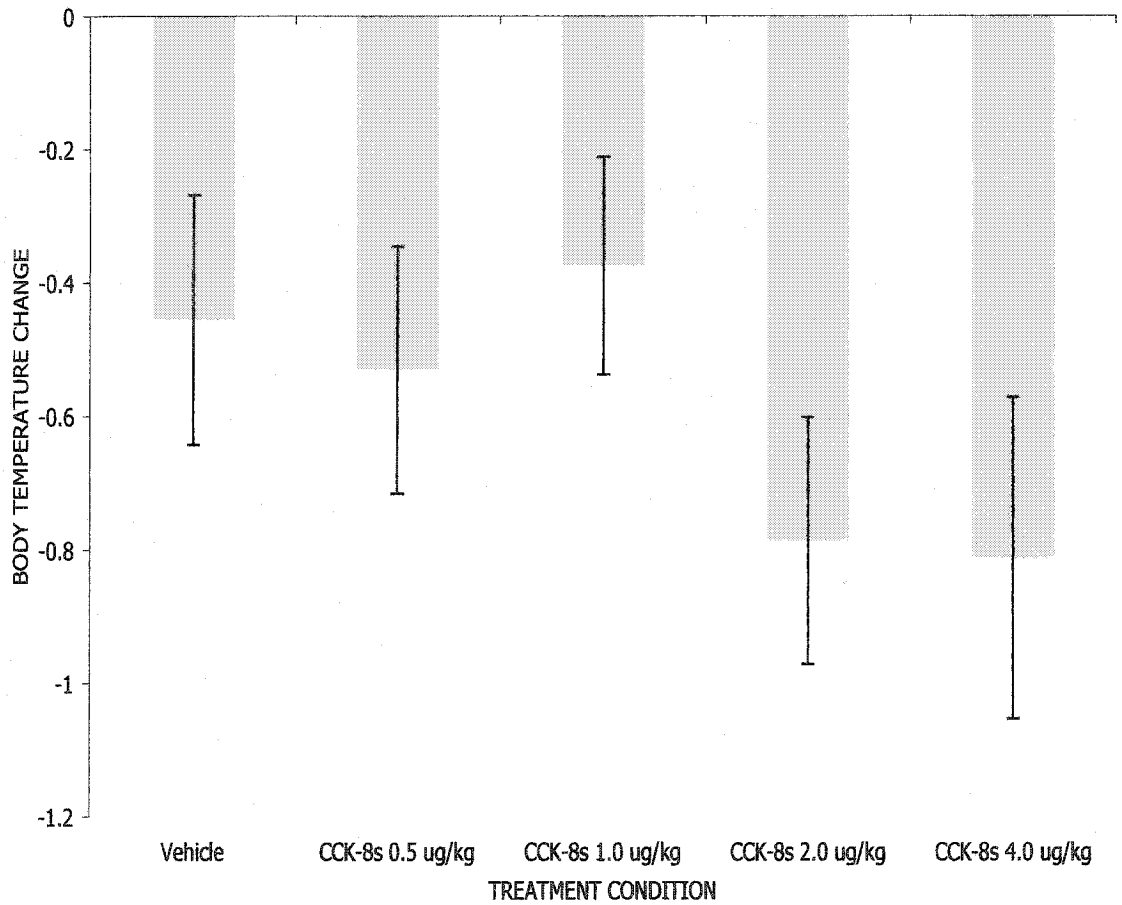


Figure 5f: Mean (±S.E.M.) Body Temperature Change following pre-treatment with vehicle or CCK-8s, administered 15 minutes prior to behavioural testing.

2.3.4. Experiment 4: CCK-8s (30 Minute Pre-treatment) Modulation of UVs, Motor Activity, and Body Temperature

2.3.4.1. Ultrasonic Vocalizations

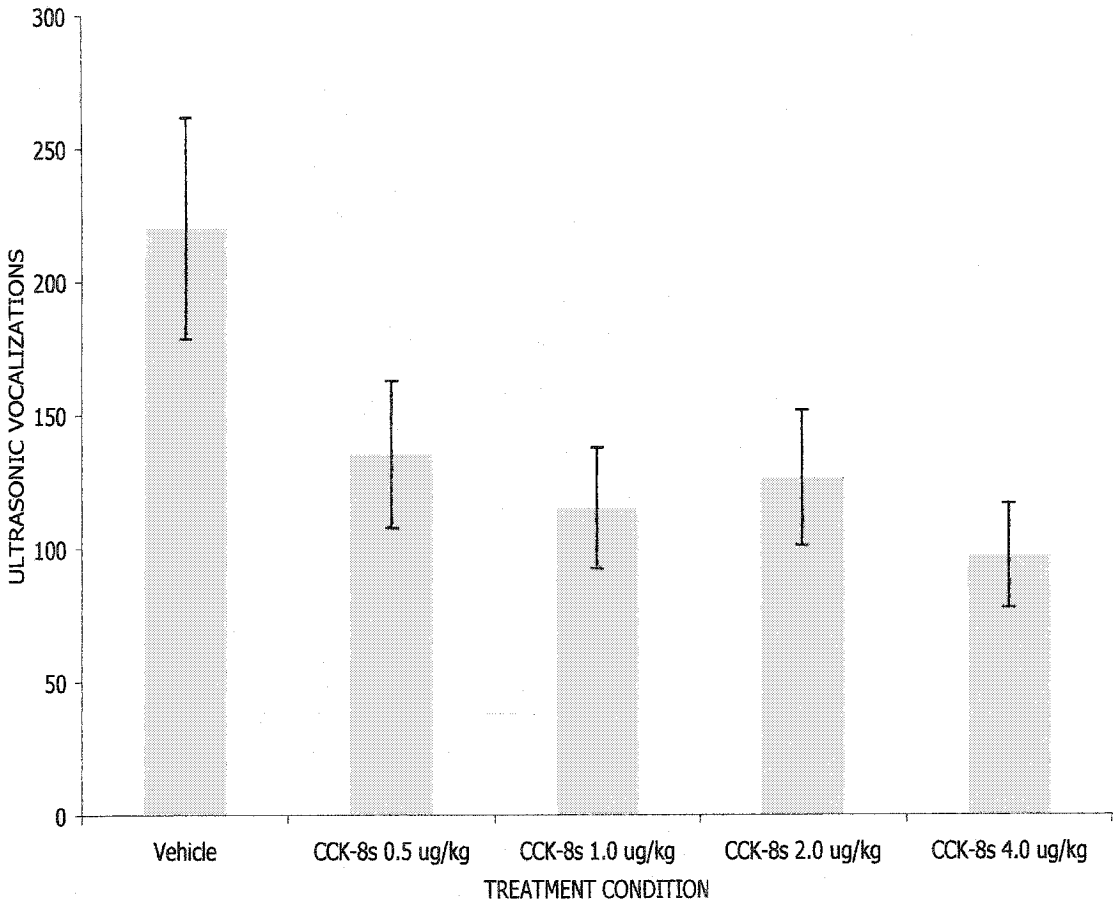
Results of the ANOVA conducted on the UV data revealed a statistically significant main effect ($F_{4,75} = 2.819$, $p = .0309$) for CCK-8s Dose (Figure 6a). Post hoc multiple comparisons indicated that when administered 30 minutes before testing, each of the four doses of CCK-8s (0.5 μg/kg, 1.0 μg/kg, 2.0 μg/kg, and 4.0 μg/kg) significantly reduced the rate of UVs compared to vehicle.

2.3.4.2. Motor Activity

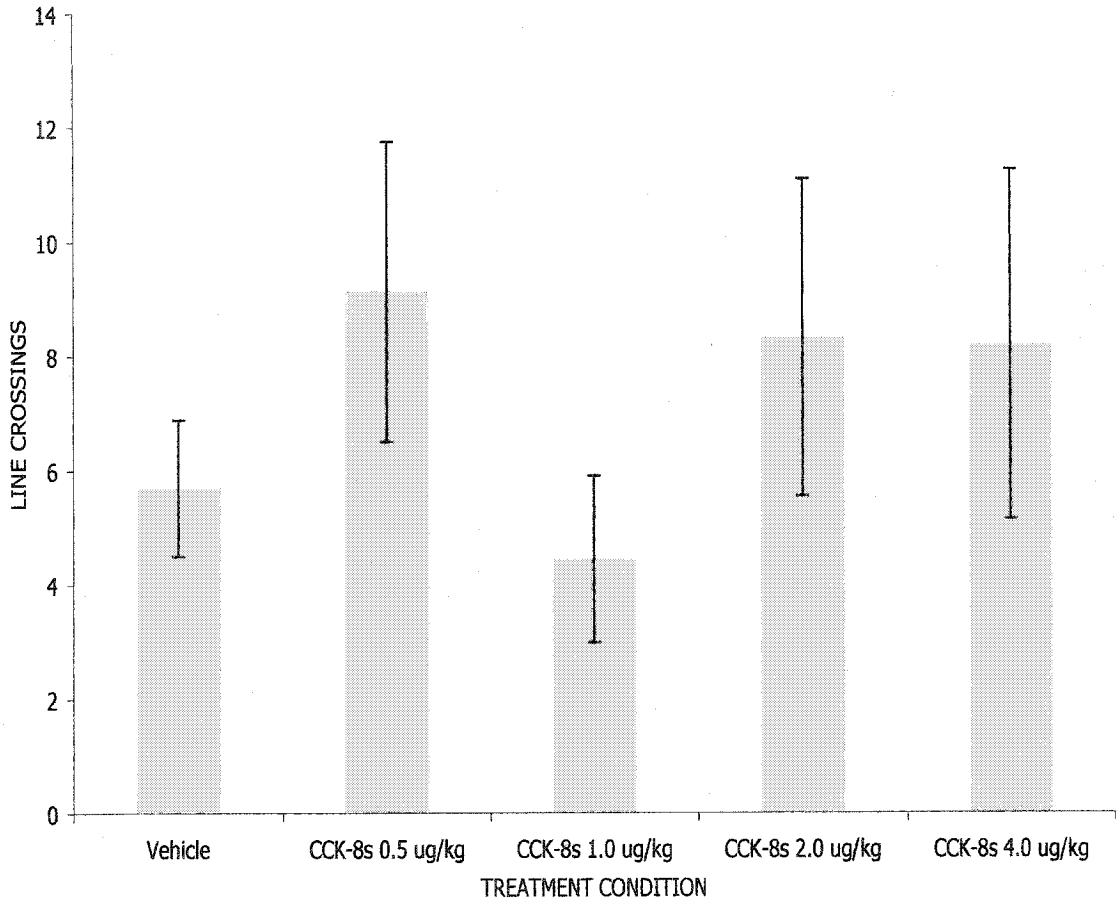
In contrast to its effect on UVs, CCK-8s did not exert a significant influence on rates of Line Crossing ($F_{4,75} = .722$, $p = .5797$; Figure 6b), Turns ($F_{4,75} = .178$, $p = .9490$; Figure 6c), Head Raises ($F_{4,75} = .323$, $p = .8619$; Figure 6d), or Wall Climbing ($F_{4,75} = 1.066$, $p = .3793$; Figure 6e).

2.3.4.3. Body Temperature Change

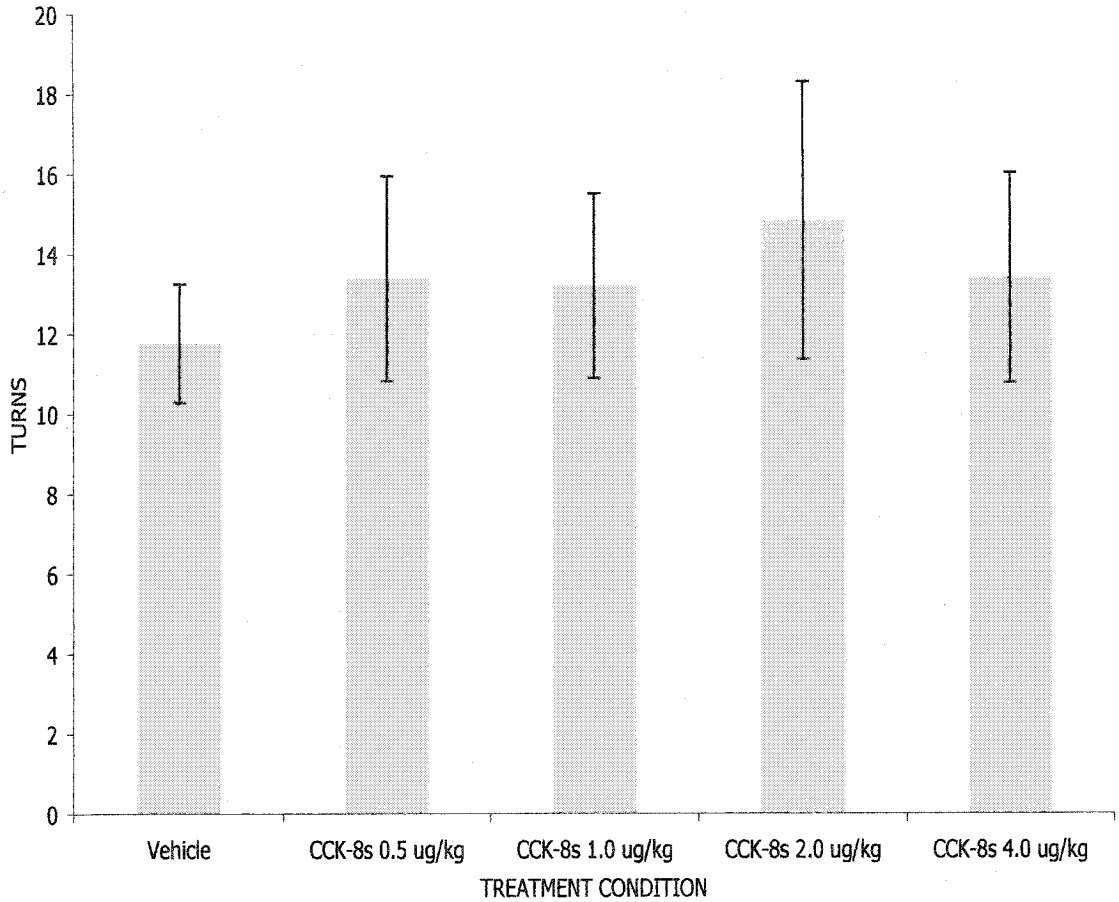
When administered 30 minutes before testing, CCK-8s also failed to significantly influence body temperature ($F_{4,75} = 2.275$, $p = .0690$; Figure 6f).



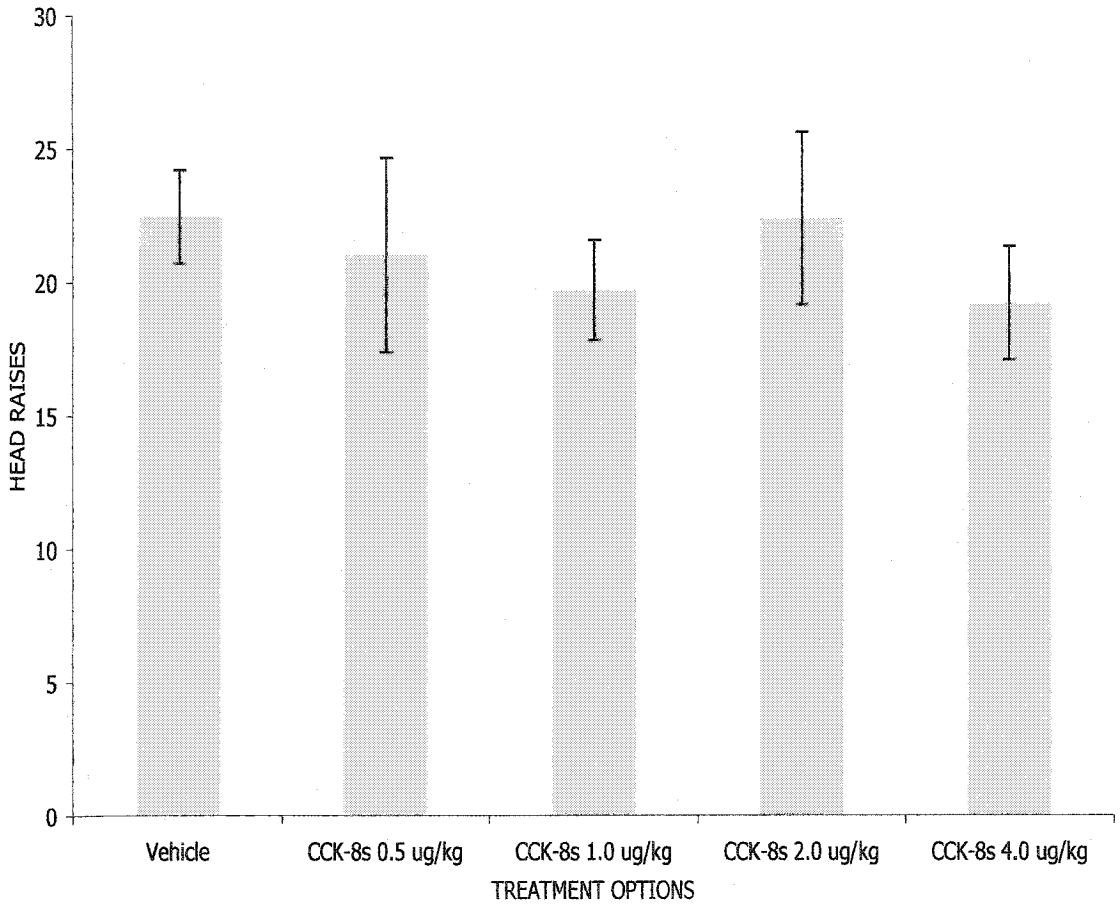
Figures 6a: Mean (±S.E.M.) UV rates over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 30 minutes following drug pre-treatment.



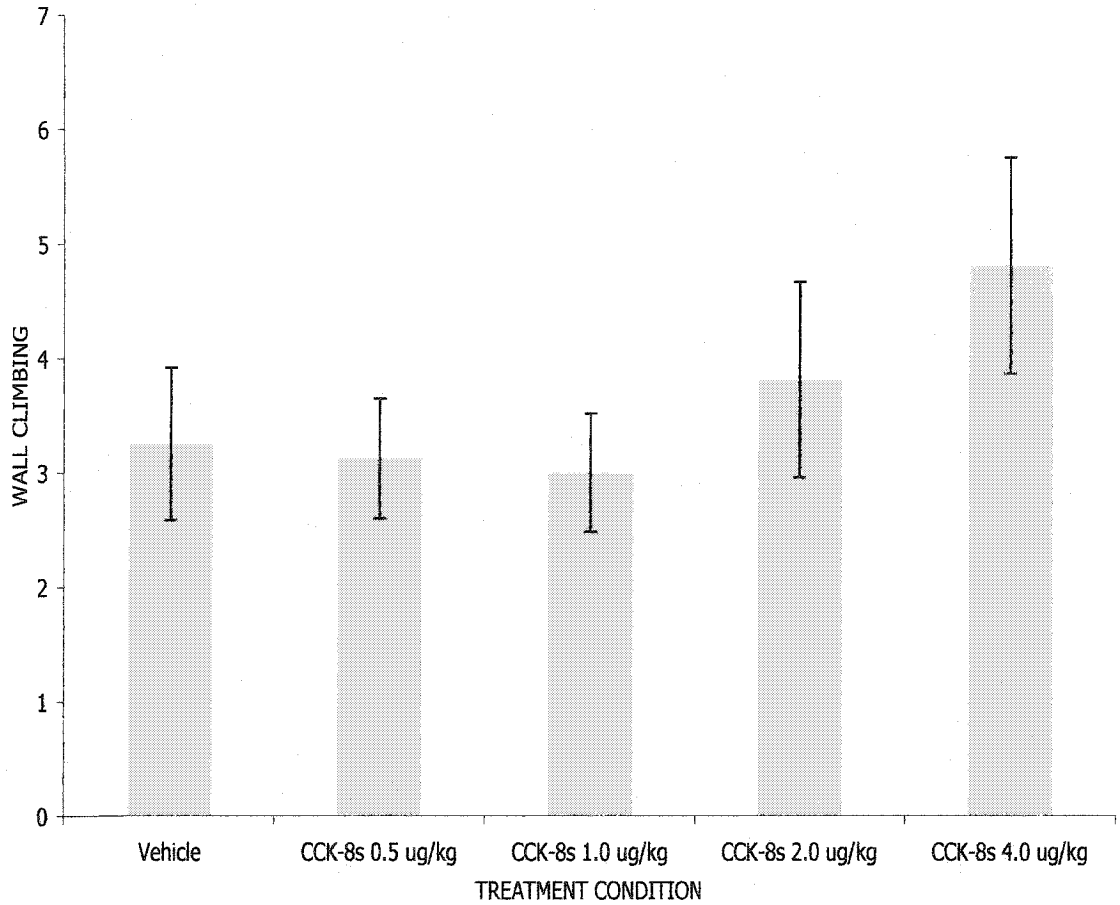
Figures 6b: Mean (±S.E.M.) Line Crossing rates over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 30 minutes following drug pre-treatment.



Figures 6c: Mean (±S.E.M.) rates of Turning over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 30 minutes following drug pre-treatment.



Figures 6d: Mean (±S.E.M.) rates of Head Raises over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 30 minutes following drug pre-treatment.



Figures 6e: Mean (±S.E.M.) Wall Climbing rates over a 6-minute period, following treatment with vehicle or CCK-8s. Behavioural testing was conducted 30 minutes following drug pre-treatment.

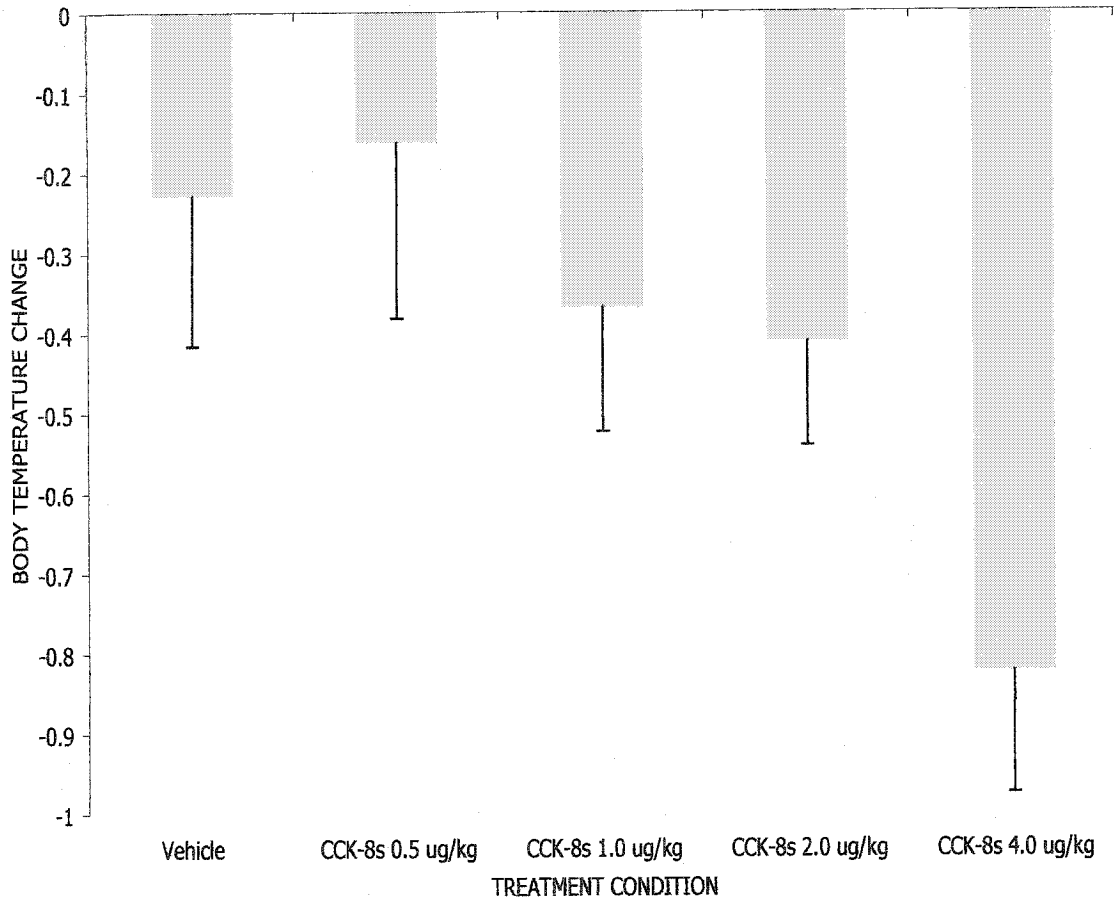


Figure 6f: Mean (±S.E.M.) Body Temperature Change following pre-treatment with vehicle or CCK-8s, administered 30 minutes prior to behavioural testing.

2.4. Discussion: Experiments 1-4

Systemic pretreatment of 12-day-old rat pups with BOC-CCK-4 failed to affect rates of isolation-induced UVs, line crossing, turning, head raises, wall climbing, or body temperature. These findings are in contrast to the results of Rex et al. (1994), who reported a dramatic increase in UV rates, following intraperitoneal pretreatment with 10 μg/kg BOC-CCK-4.

Several significant methodological differences between these studies may, in part, account for these discrepant findings. The strains of rats used in the two studies differed (Long Evans hooded versus Wistar). Strain differences have been reported with respect to isolation-induced UV rates in rat pups (Nitschke et al., 1975; Sales, 1979). In particular, Wistar rat pups tend to be "more vocal" compared to hooded rats, in that the former typically emit louder and more frequent UVs when isolated or handled.

Another difference between the current experiments with BOC-CCK-4 and the experiment conduced by Rex et al. (1994) is that they tested 5-day-old pups, whereas in the current experiments, 12-day-old pups were used. Central CCK receptors develop rapidly during the first two postnatal weeks (Hays et al., 1981). Although relatively few central receptors exist in 5-day-old pups, by 12 days of age, central CCK receptor development has reached a plateau. As such, the receptor system on which BOC-CCK-4 acted was markedly different across the two studies. Given that CCK interacts with numerous other neurochemical systems (Crawley & Corwin, 1994), one must also consider the developmental

stage of other neurochemical systems as well when attempting to understand the behavioural effects (or lack thereof) of BOC-CCK-4. Evidence supporting the potential contribution of developmental factors in terms of how CCK system activation affects UVs can be gleaned from above noted studies involving CCK-8s, and the current data from Experiment 4. Specifically, CCK-8s did not affect UVs in 5- to 9-day-old pups (Rex et al.; Weller & Dubson, 1998) but was anxiolytic in 11-day-old (Weller & Blass, 1988), and 12-day-old (current data from Experiment 4) pups. Again, the potential impact of developmental factors must be considered.

Vocalization rates among 5-day-old rat pups are typically low when pups are tested at temperatures closer to those of the nest (as in the case of the Rex et al. [1994]). At this age, thermal cues are the primary eliciting factors for UVs, whereas in older pups (i.e., 12 days of age), thermal cues become less salient (Hofer, 1996). Given that Rex and colleagues failed to measure body temperature changes in response to BOC-CCK-4, it is not possible to demonstrate that the "anxiogenic" effects they reported were not actually due to changes in body temperature induced by BOC-CCK-4. This point is especially relevant given that CCK has been shown to induce hypothermia in the rat. In addition, Rex et al. did not measure motor activity, which is also affected in CCK (Crawley & Corwin, 1994). As such, no conclusions can be drawn from these data as to whether CCK-4 exerted a selective effect on anxiety-related behaviour.

In the current study, BOC-CCK-4 did not affect body temperature, which suggests that the 12-day-old pups were able to cope adequately with the thermal demands associated with isolation at room temperature. The lack of effect on motor activity indicates that pups were not sedated or motorically incapacitated to a degree where vocalization was not possible. Finally, the fact that pups did not vocalize in response to BOC-CCK-4 could not be attributed to a ceiling effect associated with control group UVs. Inspection of the data revealed that the mean number of control group UVs was 360 after 15 minutes pre-treatment, and 150 after 30 minutes pre-treatment. Some individual pups in this series of studies emitted much higher rates of UVs, indicating that increased calling above the mean control values was possible.

It will be recalled that CCK-8s administered 15 minutes prior to testing has been shown to either decrease (Weller & Blass, 1988) or to have no effect (Rex et al., 1994; Weller & Dubson, 1998) on UVs. In the current study, CCK-8s was also without effect after 15 minutes. The reason(s) for lack of a significant effect associated with CCK-8s administered 15 minutes before testing in the current study is not readily apparent. However, CCK-8s administered 30 minutes prior to testing reduced UVs across all of the doses examined, and these effects were specific in that motor behaviour and body temperature were not affected. Of note, Weller & Blass (1988) also tested the effects of 5.0μg-20μg of CCK-8s from 5 to 25 minutes after pre-treatment and found that the anxiolytic effects on UVs did not differ significantly after the more protracted pre-treatment interval (i.e.,

the anxiolytic effect was equally present across the intervals). These anxiolytic effects of CCK-8s may be mediated by either CCK-1 or CCK-2 receptors, given that both receptor subtypes exhibit a relatively similar degree of affinity for CCK-8s. However, given that BOC-CCK-4 was without effect under the exact same testing circumstances in the current set of experiments, a role for the CCK-2 receptor is questionable. Experiments 5 and 6 were therefore designed in order to: a) assess the intrinsic effects of CCK-1 and CCK-2 receptor antagonists on UVs, motor behaviour, and body temperature, and b) determine whether antagonists of either/both receptor subtypes could prevent the anxiolytic effects of CCK-8s.

3.0. EXPERIMENTS 5-6: BLOCKADE OF THE EFFECTS OF CCK-8s WITH DEVAZEPIDE OR L-365,260

3.1. Introduction: Experiments 5-6

As in the case of the CCK agonists, few studies have examined the effects of CCK antagonists on UVs, and within this body of research, emphasis has clearly been placed on CCK-1, as opposed to CCK-2, antagonists. With respect to the former, devazepide (1mg/kg) failed to exert any intrinsic effects on isolation-induced UVs in 6- to 9-day-old pups when administered 15 minutes prior to testing (Weller & Dubson, 1998) or in 10- to 11-day-old pups 30 minutes following pretreatment (Blass & Shide, 1993; Weller & Gispan, 2000). Devazepide did, however, significantly increase rates of head raising and wall climbing, according to Weller and Dubson (1998), although other aspects of

motor activity (i.e., line crossing, self-grooming, rolling over, or pivoting) were not significantly affected by the CCK-1 antagonist.

While antagonism of CCK-1 receptors appears to be without effect on isolation-induced UVs in otherwise untreated pups, devazepide does reverse the reductions in UV rates that occurs during active milk consumption (Weller & Gispan, 2000), or following intra-oral milk or corn oil infusion (Blass & Shide, 1993). In contrast, UV reductions following treatment with intra-oral sucrose are not blocked by devazepide pre-treatment. Of note, milk or corn oil, but not sucrose intake, has been associated with endogenous CCK release (Blass & Shide, 1993). These findings indicate a specific role for CCK-1 receptor antagonism in blocking UV reductions associated with at least some stimuli that provoke endogenous CCK release. Moreover, consistent with the reported divergence between the effects of exogenous CCK-8s on UVs and analgesia (Weller & Blass, 1988), devazepide does not block milk- or fat-induced analgesia, demonstrating that experimental manipulations involving CCK exert specific behavioural effects in pups. Taken together, these findings would suggest that at least some experimental manipulations that trigger endogenous CCK release (i.e., milk or fat consumption) are associated with decreased UVs, and this effect is mediated by CCK-1 receptors.

It will be recalled that re-uniting pups with the dam provokes an increase in plasma CCK concentrations (Weller et al., 1992), and a decrease in UVs (Hofer et al., 1993b; Hofer & Shair, 1978, 1987; Shair et al., 1999). Based on the above

noted findings involving milk and corn oil, it would be expected that the decrease in UV rates following presentation of the dam to a pup might be attenuated by devazepide pre-treatment. Consistent with this prediction, Weller and Dubson (1998) found that when 6- to 9-day-old Sprague-Dawley pups were treated with devazepide and then placed in a cage with an awake dam, the pups responded with elevated UV rates (compared to vehicle- or CCK-8s-treated pups). Dams responded, in turn, with increased maternal care directed towards the pup (in the form of amount of time spent near the pup and direct hovering over the pup). However, in contrast to these findings, 10- to 11-day-old Sprague-Dawley pups that were administered devazepide responded to an anesthetized dam with a "standard" decrease in call rates (Weller & Gispan, 2000). In an attempt to reconcile these discrepant findings, Weller and Gispan (2000) speculated that that in the case of the awake dam, the increase in pup UV rates might not have been the direct result of devazepide's antagonism of CCK-1 receptors. Rather, manipulation of the CCK system might have resulted in behavioural and/or olfactory changes in the pup, which in turn could have altered the manner in which the dam interacted with the pup.

Taken together, the data regarding devazepide's effects on UVs suggests that blockade of the CCK-1 receptor modulates the quieting effects of milk and fat (and perhaps contact quieting associated with presentation of an awake dam). In contrast, antagonism of the CCK-1 receptor does not affect isolation-induced UVs. That being said, Weller, Avnon, Hurwitz, and Malkesman (2002)

reported that rates of isolation-induced UVs were significantly elevated in Otsuka Long Evans Tokushima Fatty (OLETF) rat pups, which do not express the CCK-1 receptor, because of a genetic abnormality. Lack of CCK-1 receptors rather than simple blockade of CCK-1 receptors with devazepide, may have resulted in developmental changes affecting other neurochemical systems, which in turn resulted in higher UV rates in OLETF rat pups under considerations of isolation. When considering the failure of devazepide to alter isolation-induced UVs in pups that possess an intact CCK-1 receptor system, it is important to note that blockade of CCK-1 receptors under such conditions may have no impact on UV rates because the endogenous CCK system is already in a state of compromise, owing to low endogenous levels of the peptide secondary to isolation (Weller et al., 1992). In other words, if endogenous CCK levels are already significantly diminished as a result of isolation, the added antagonism of CCK-1 receptors may not yield any significant changes to UVs, due to what could be termed as a "neurochemical floor effect". As such, administration of devazepide in conjunction with CCK-8s might be a more appropriate method of studying the role of the CCK-1 receptor under conditions of isolation.

With respect to devazepide's apparent lack of effect on the decrease in pup UVs observed following presentation of an anesthetized dam, the data from Hofer and Shair (1980) clearly demonstrate that pup UV rates are influenced by numerous sensory cues. Information associated with such cues is likely relayed to the brain via multiple neurochemical systems (Hofer, 1996). As such,

effectively "removing" the influence of CCK-1 receptors (via devazepide administration) from the overall neuromodulatory matrix that regulates UVs under conditions of re-unification with the dam might not be sufficient to significantly alter UV rates. Perhaps sensory information transmitted via other neurochemical systems remains sufficient to maintain the calming effect of the dam, and thus to prevent a reversal of the associated decrease in call rates.

It will be recalled that both CCK-1 and CCK-2 receptors display an affinity for CCK-8s (Crawley & Corwin, 1994). Therefore, it is difficult to determine which receptor subtype mediated the reduction in UVs noted to occur following CCK-8s administration in Experiment 4. Given that BOC-CCK-4 failed to exert any significant effects in Experiments 1 or 2, it seems that CCK-2 mediation of the CCK-8s effects is unlikely. However, data regarding the combined effects of CCK fragments and antagonists on UVs are lacking, and there are no studies that examine the effects of CCK-2 antagonists on isolation-induced UVs.

The following experiments were designed in order to assess the intrinsic effects of CCK-1 and CCK-2 receptor antagonists on isolation-induced UVs, motor activity, and body temperature, and to determine whether pre-treatment with either type of CCK receptor antagonist could block the reduction in UVs associated with CCK-8s, as detected in Experiment 4. Based on previous data (see above) it was hypothesized that antagonism of the CCK-1 receptor would not significantly affect isolation-induced UV rates. Given that BOC-CCK-4 did not significantly affect UVs in Experiments 1 and 2, it was also hypothesized that

CCK-2 receptor antagonism would not have an effect on UVs when administered on its own. In addition, the findings from Experiments 1 and 2 would suggest that CCK-8s did not exert its effects via the CCK-2 receptor. As such, it was predicted that CCK-1, but not CCK-2 antagonism would block the anxiolytic effects of CCK-8s.

3.2. Method: Experiments 5-6

3.2.1. Subjects

Subjects used in experiments 5 and 6 were offspring of Long Evans hooded rats, purchased from Charles River Canada (St. Constant, Quebec). The general mating and rearing procedures were identical to those outlined for Experiments 1 to 4. Experiment 5 had 120 subjects, with 12 subjects randomly assigned to each of 10 treatment conditions. Experiment 6 initially had 90 subjects, with nine subjects randomly assigned to each of 10 treatment conditions. However, the data for one subject was lost due to experimenter error, leaving one of the five treatment conditions with only 8 subjects, for a total of 89 subjects. Only litters culled to 10 pups were used, in order to ensure equal distribution of littermates across treatment conditions. Pups remained with the dam until the day of testing (Day 12). Behavioural testing was conducted during the subjects' dark cycle.

3.2.2. Drugs

The CCK-1 receptor antagonist, 3S-(-)-(1,3 dihydro-3-(2-indole-carbonyl) amino-1-methyl 5-phenyl-2H-1,4-benzodiazepin-2-one) (Devazepide), and the

CCK-2 receptor antagonist, 3R-3-[N'-(3-methylphenyl) ureido]-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (L-365,260), were generously donated by Dr. Les Iverson and ML Laboratories (Oxford, U.K.). Both devazepide and L-365, 260 were sonicated for 10 minutes, in vehicle, which consisted of two drops of Tween 80, sonicated in 10 μl of distilled water (Adamec, Shallow, & Budgell, 1997; Johnson & Rodgers, 1996).

CCK-8s (Sigma Pharmaceuticals, St. Louis, U.S.A) was dissolved in distilled water. All injections were administered i.p. in a 2ml/kg volume, with a 100 μl syringe (Becton-Dickenson & Co., Franklin Lakes, N.J., U.S.A.).

3.2.3. Apparatus

The measurement of UVs, motor activity (Line Crossings, Turning, Head Raises, and Wall Climbing), and body temperature was conducted using the same equipment described in Experiments 1-4.

3.2.4. Procedure

On postnatal Day 12 subjects were transported in their homecage with the dam, from the colony room to an adjacent testing room and left undisturbed for a 30-minute acclimatization period. Following the acclimatization period, a single subject was removed from the homecage and transported to the testing room in a small container containing homecage bedding. Each subject was weighed and its pre-first injection body temperature was recorded.

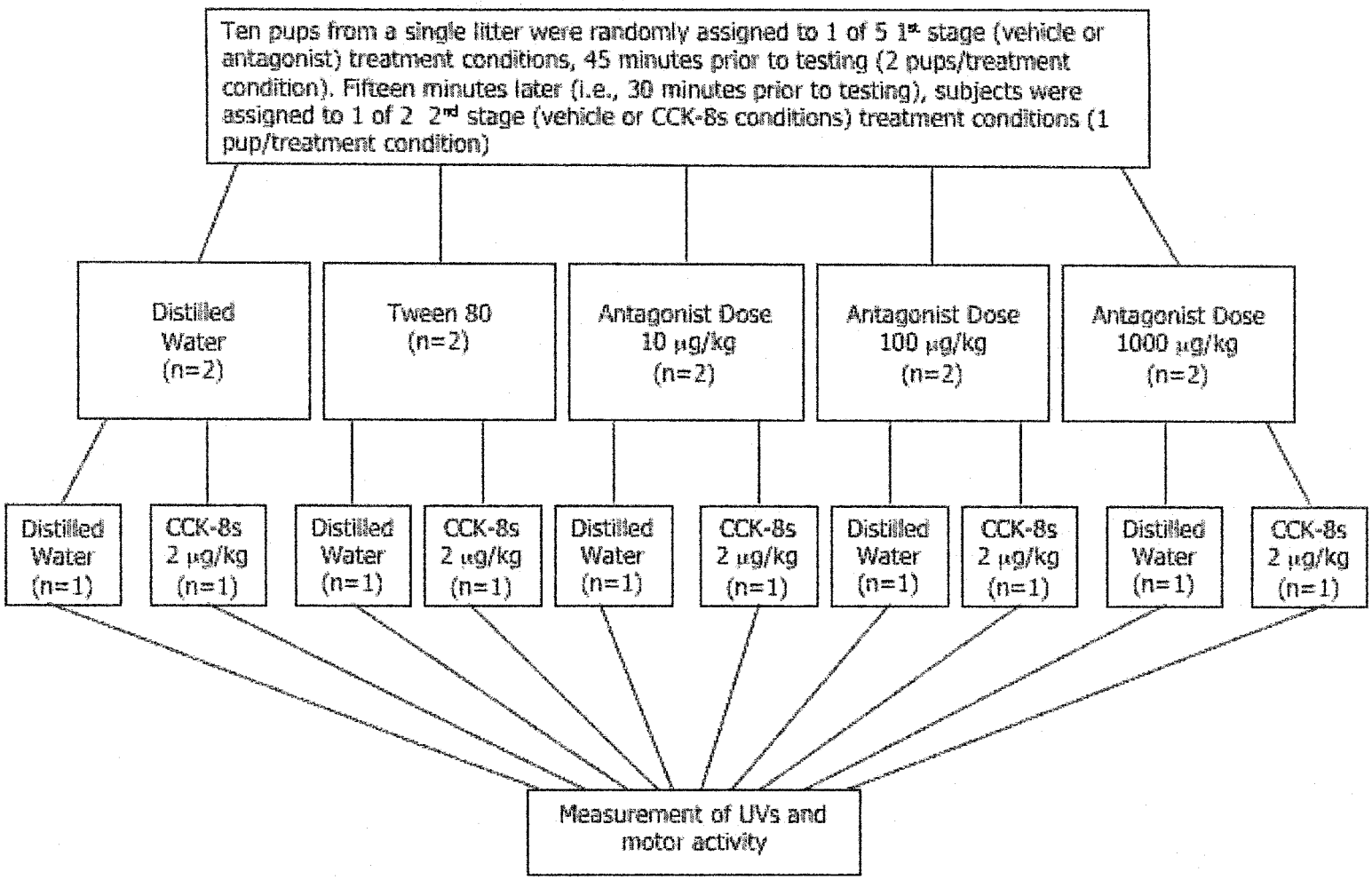
Experiments 5 and 6 each consisted of 10 treatment conditions. In each experiment, a split-litter design was employed, with one of the ten pups from each litter randomly assigned to one of the 10 treatment conditions.

Forty-five minutes prior to testing, each subject received an initial injection of distilled water, Tween 80 vehicle, one of three doses of devazepide (10 μg/kg, 100 μg/kg, or 1000 μg/kg; Experiment 5), or one of three doses of L-365,260 (10 μg/kg, 100 μg/kg, or 1000 μg/kg; Experiment 6). Following this initial injection, the subject was placed in a holding cage, which was partially submerged in a warm water bath maintained at 35 ±1°C, in order to simulate the ambient temperature of the nest. In both Experiments 5 and 6, fifteen minutes following the initial injection, each subject was removed from the holding cage and its pre-second injection body temperature was recorded. Subjects were then injected with either distilled water vehicle or 2.0 mg/kg of CCK-8s (see Figure 7 for a breakdown of how subjects from each litter were assigned to one of the 10 treatment conditions). The subject was then returned to the holding cage.

Thirty minutes following the second injection, the subject was then transported to the testing room and placed in the centre of the testing chamber. Rates of UVs, line crossing, turning, rearing, and wall climbing, were recorded over a 6-minute period. The ambient temperature was 22 ±1°C.

Following the conclusion of the 6-minute test period, each subject was removed from the test chamber and the post-test body temperature was

Figure 7: Assignment of subjects to one of ten different treatment conditions in Experiments 5 and 6



recorded. The subject was then placed in a cage separate from the dam and untreated littermates. Each subject from every litter was tested on one occasion only.

3.2.5. Data Screening and Statistical Analyses

The same screening techniques that were used in Experiments 1-4 were also used to assess for outliers, normal distributions, and homogeneity of variance in Experiments 5 and 6 (see above for full explanation and Table 2 for a breakdown of outliers in each of the treatment groups, across each of the dependent variables).

In Experiments 5 and 6, rates of UVs, Line Crossings, Turns, and Wall Climbing were analyzed using a series of 5 x 2 ANOVAs. The first between group factor in Experiment 5 was the Devazepide Dose, which consisted of five levels (distilled water, Tween 80, and three doses of devazepide). The first between group factor in Experiment 6 was the L-365,260 Dose, which also had five levels (distilled water, Tween 80, and the three doses of L-365,260). The second between group factor was identical in Experiments 5 and 6, and was the CCK-8s Dose, which consisted of two levels (vehicle and CCK-8s, 2.0 mg/kg).

Changes in body temperature were calculated using the same method employed in Experiments 1-4. In Experiment 5, these data were analyzed with a 5 x 2 ANOVA, with Devazepide Dose and CCK-8s Dose as the between group factors. In Experiment 6, a 5 x 2 ANOVA was used with L-365,260 Dose and CCK-8s Dose as the between group factors.

Table 2: Breakdown of outliers in Experiments 5 and 6, across each of the dependent variables. Values within each cell of the tables represent one case, expressed as the number of SDs away the subject's score fell from the treatment group mean.

Experiment 5: Devazepide Dose/CCK-8s 2.0 μg/kg pre-treatment*

	Line Crossings	Turns	Head Raises	Wall Climbing	UVs	Body Temperature Change
W/W	2.171	-	-	2.933	2.344	2.220
W/CCK-8s	2.070	-	2.316	2.266	-	-
T/W	2.519	-	-	2.297	-	2.484
T/CCK-8s	3.040	2.181	2.396	2.253	2.310	2.211
10/W	2.678	-	-	-	2.480	-
10/CCK-8s	2.517	-	-	2.039	-	-
100/W	2.255	-	-	-	-	-
100/CCK-8s	2.963	2.551	-	-	2.495	-2.065
1000/W	2.285	-	-	-	-	-
1000/CCK-8s	2.422	-	2.077	-	-	-

*W=Distilled Water; T=Tween; all doses of L-365,260 (10, 100, 1000) are in μg/kg units.

Table 2: (continued)

Experiment 6: L-365,260 Dose/CCK-8s 2.0 μg/kg pre-treatment*

	Line Crossings	Turns	Head Raises	Wall Climbing	UVs	Body Temperature Change
W/W	2.184	-	-	2.568	2.621	-2.168
W/CCK-8s	-	-	-	2.162	2.163	-
T/W	2.513	-	-	2.587	-	2.182
T/CCK-8s	2.455	2.147	-	2.179	-	2.206
10/W	-	2.093	-	-	-	-
10/CCK-8s	2.593	2.059	-	-	2.284	2.367
100/W	-	2.012	2.149	-	-	-
100/CCK-8s	2.576	-	-	2.007	2.318	-
1000/W	-	2.228	2.341	2.236	-	2.227
1000/CCK-8s	2.162	-	-2.214	2.113	2.085	-

***W=Distilled Water; T=Tween; all doses of L-365,260 (10, 100, 1000) are in μg/kg units.**

Significant interactions between Devazepide or L-365,260 Dose and CCK-8s Dose were followed up with a series of paired t-tests, comparing the effects of CCK-8s- versus distilled water-treated pups, across the different dose levels of the antagonist.

3.3. Results: Experiments 5-6

3.3.1. Experiment 5: Blockade of the Effects of CCK-8s (30 minute pre-treatment) with Devazepide (45 minute pre-treatment)

3.3.1.1. Ultrasonic Vocalizations

The CCK-1 antagonist devazepide, failed to significantly influence the production of UVs ($F_{4,110}$ = .526, p = .7166). In addition, the main effect of CCK-8s Dose was not replicated in the current experiment ($F_{1,110}$ = .047, p = .8288). Finally the interaction between devazepide and CCK-8s also failed to reach the necessary level of statistical significance ($F_{4,110}$ = 1.335, p = .2616; Figure 8a).

3.3.1.2. Motor Activity

Neither devazepide nor CCK-8s induced a significant influence on any of the motor behaviours assessed in the current experiment. Specifically, the ANOVA associated with the variable Line Crossings yielded no significant effect for Devazepide Dose ($F_{4,110}$ = .858, p = .4915), CCK-8s Dose ($F_{1,110}$ = 2.164, p = .1441), or the interaction between Devazepide Dose and CCK-8s ($F_{4,110}$ = .616, p = .6518; Figure 8b).

Similarly, devazepide was without effect on Turning ($F_{4,110}$ = 1.247, p = .2952). In addition, the main effect for CCK-8s Dose was not statistically

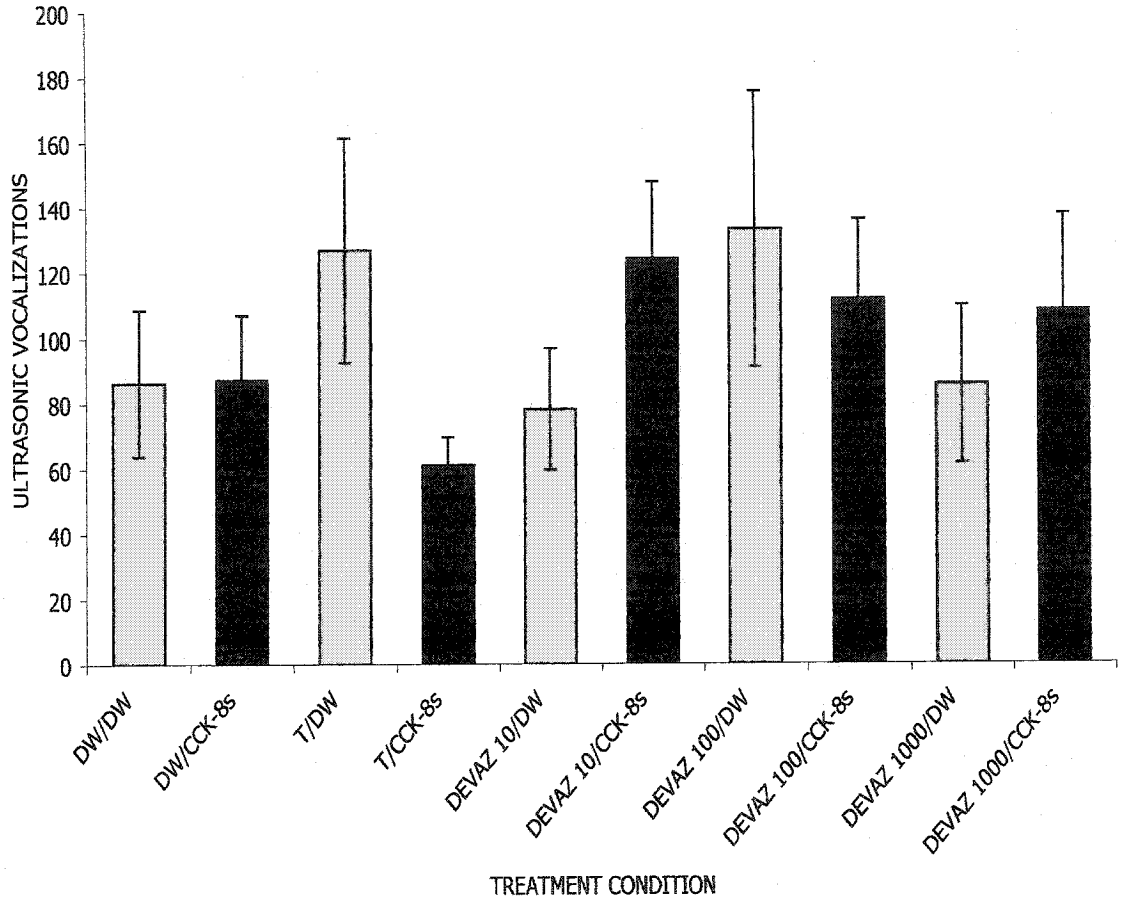
significant ($F_{1,110}$ = 1.093, p = .2982). The Devazepide Dose X CCK-8s Dose interaction also failed to reach statistical significance ($F_{4,110}$ = .555, p = .6956; Figure 8c).

The analysis associated with the Head Raises data did not detect significant main effects for Devazepide Dose ($F_{4,110}$ = 1.653, p = .1662) or CCK-8s Dose ($F_{1,110}$ = .011, p = .9164), or a significant interaction between Devazepide Dose and CCK-8s Dose ($F_{4,110}$ = .804, p = .5253; Figures 8d).

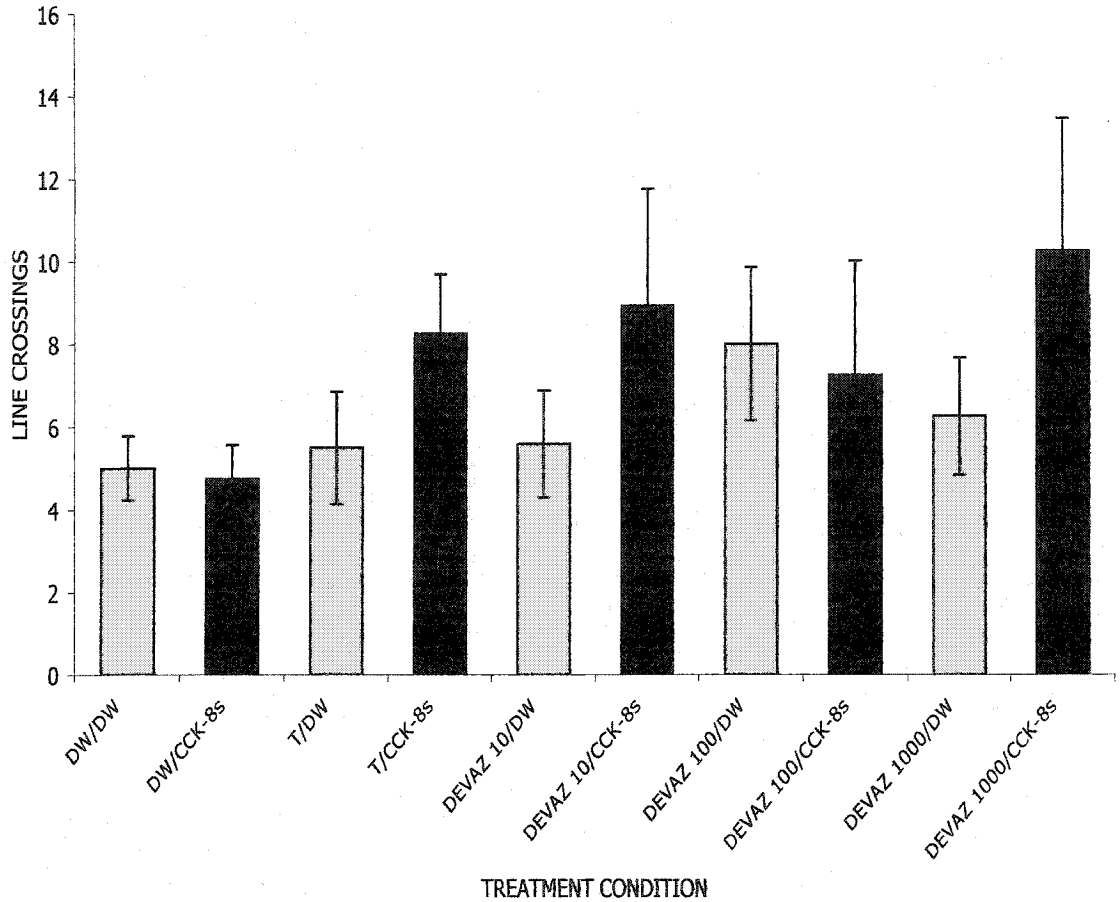
Devazepide failed to significantly alter Wall Climbing ($F_{4,110}$ = 1.660, p = .1644). Pretreatment with CCK-8s also failed to alter Wall Climbing rates ($F_{1,110}$ = .463, p = .4976). However, the interaction between Devazepide Dose and CCK-8s Dose significantly altered wall climbing rates ($F_{4,110}$ = 2.648, p = .0372; Figure 8e). Pups treated with 1000 μg/kg of devazepide and then CCK-8s exhibited significantly lower wall climbing rates compared to pups treated with 1000 μg/kg devazepide and vehicle (p=.0174).

3.3.1.3. Body Temperature Change

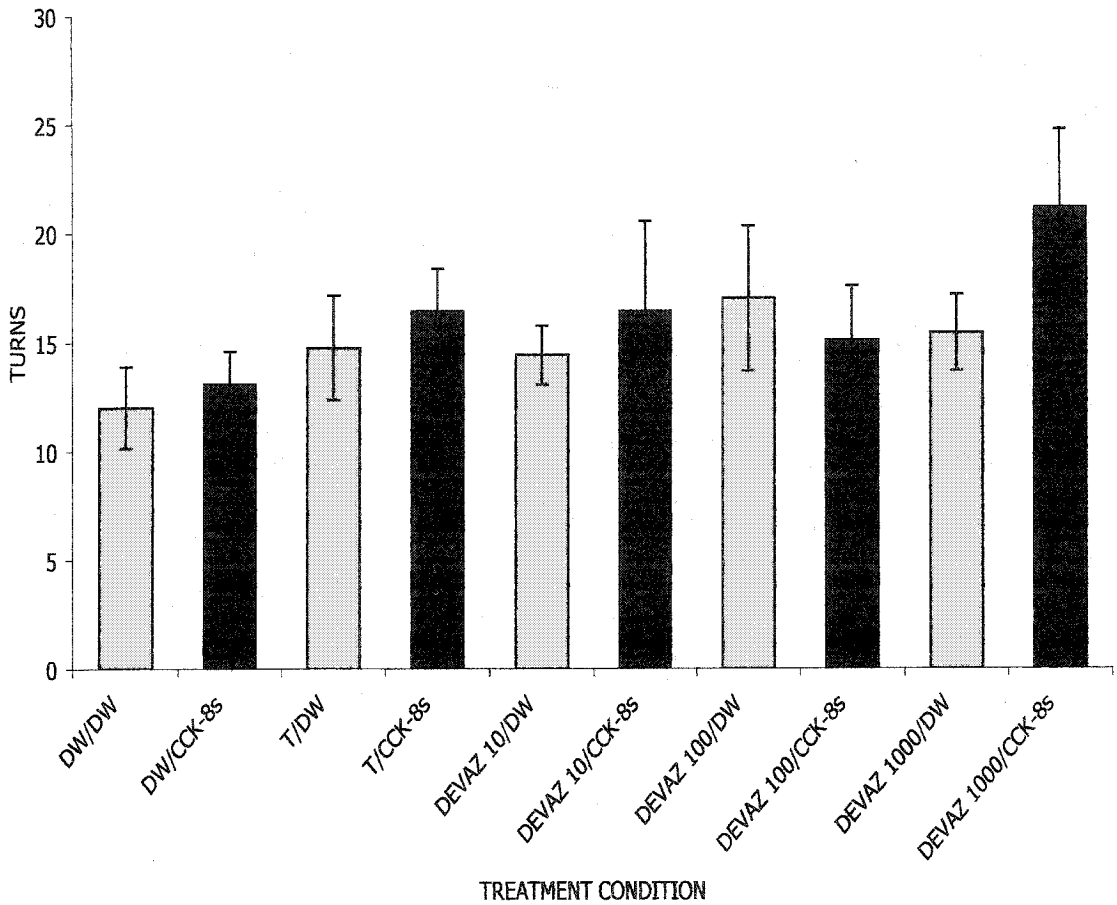
Body temperature was not significantly altered by devazepide ($F_{4,110}$ = 1.369, p = .2493) or CCK-8s ($F_{1,110}$ = .146, p = .7032), or the interaction between devazepide and CCK-8s ($F_{4,110}$ = 2.038, p = .0940; Figure 8f).



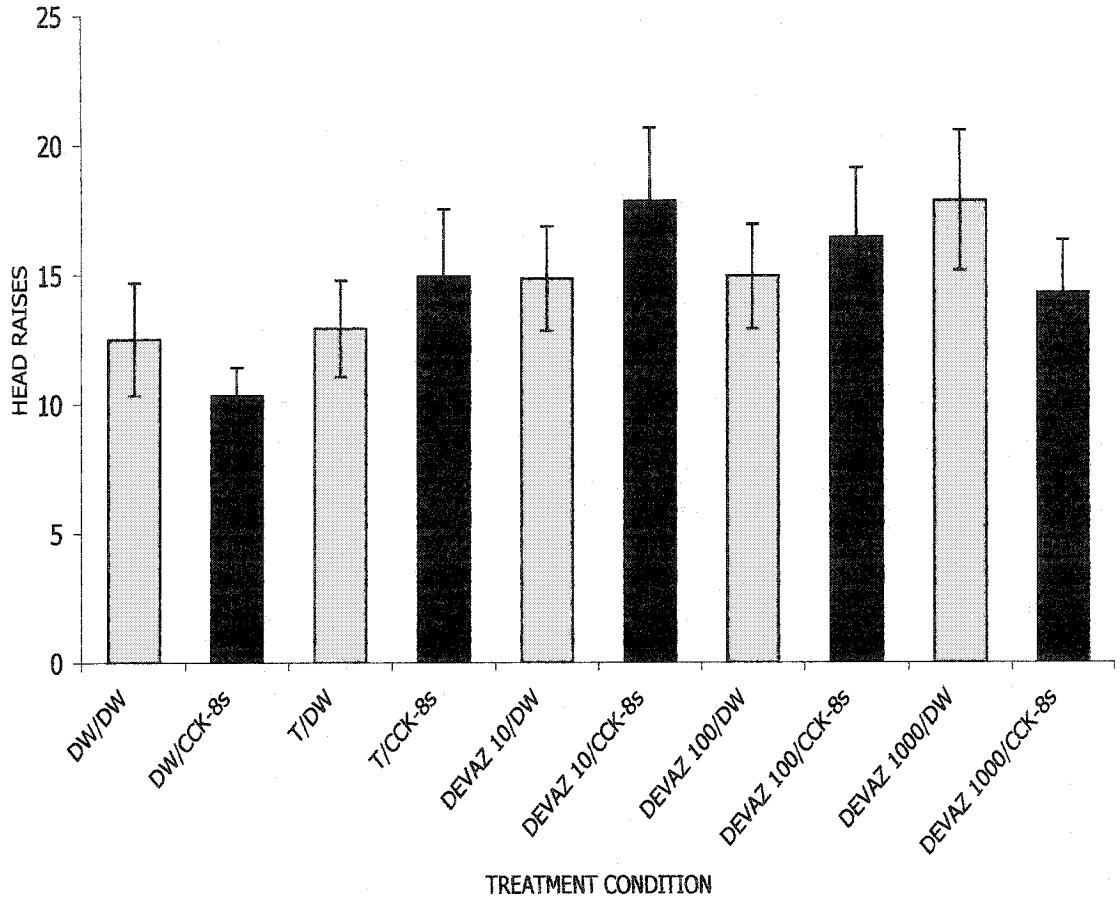
Figures 8a: Mean (±S.E.M.) UV rates across a 6-minute period, associated with the interaction between devazepide and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with devazepide (and 30 minutes following pre-treatment with CCK-8s).



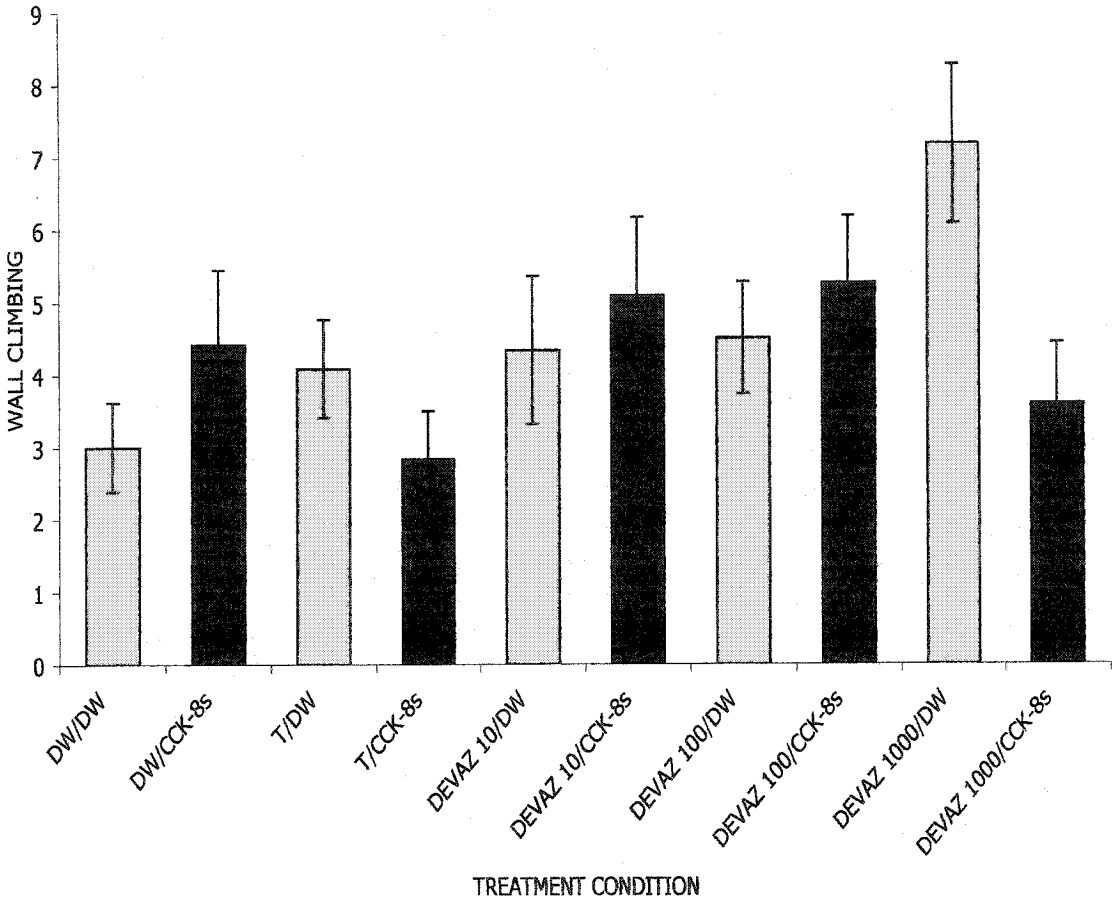
Figures 8b: Mean (±S.E.M.) rates of Line Crossing across a 6-minute period, associated with the interaction between devazepide and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with devazepide (and 30 minutes following pre-treatment with CCK-8s).



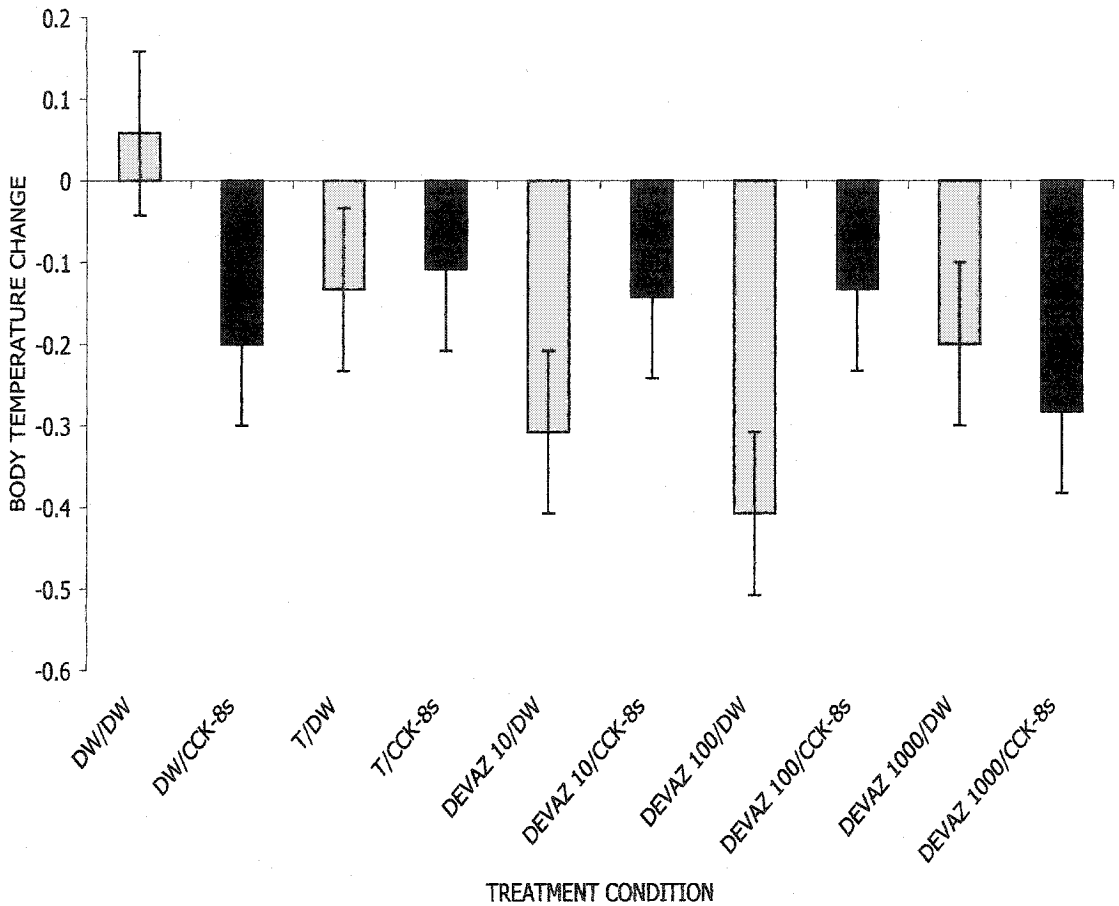
Figures 8c: Mean (±S.E.M.) Turn rates across a 6-minute period, associated with the interaction between devazepide and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with devazepide (and 30 minutes following pre-treatment with CCK-8s).



Figures 8d: Mean (±S.E.M.) rates of Head Raises across a 6-minute period, associated with the interaction between devazepide and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with devazepide (and 30 minutes following pre-treatment with CCK-8s).



Figures 8e: Mean (±S.E.M.) rates of Wall Climbing across a 6-minute period, associated with the interaction between devazepide and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with devazepide (and 30 minutes following pre-treatment with CCK-8s).



Figures 8f: Mean (±S.E.M.) Body Temperature Change across a 6-minute period, associated with the interaction between devazepide and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with devazepide (and 30 minutes following pre-treatment with CCK-8s).

3.3.2. Experiment 6: Blockade of the Effects of CCK-8s (30 minute pre-treatment) with L-365,260 (45 minute pre-treatment)

3.3.2.1. Ultrasonic Vocalizations

Neither L-365,260 ($F_{4,79}$ = .566, p = .6879), nor CCK-8s ($F_{1,79}$ = 1.925, p = .1692) exerted a significant main effect on UV rates. In addition, the interaction between L-365,260 and CCK-8s was not statistically significant ($F_{4,79}$ = .175, p = .9508; Figure 9a).

3.3.2.2. Motor Activity

Line Crossings were not significantly affected by L-365,260 ($F_{4,79}$ = .990, p = .4180), or CCK-8s ($F_{1,79}$ = .683, p = .4110). However, the interaction between L-365,260 and CCK-8s was found to significantly alter the rate of line crossings ($F_{4,79}$ = 2.539, p = .0463; Figure 9b). Specifically, pups treated with L-365,260 (10 μg/kg) and CCK-8s, engaged in fewer line crossings compared to pups that were administered L-365,260 (10 μg/kg) and vehicle (p=.0445)

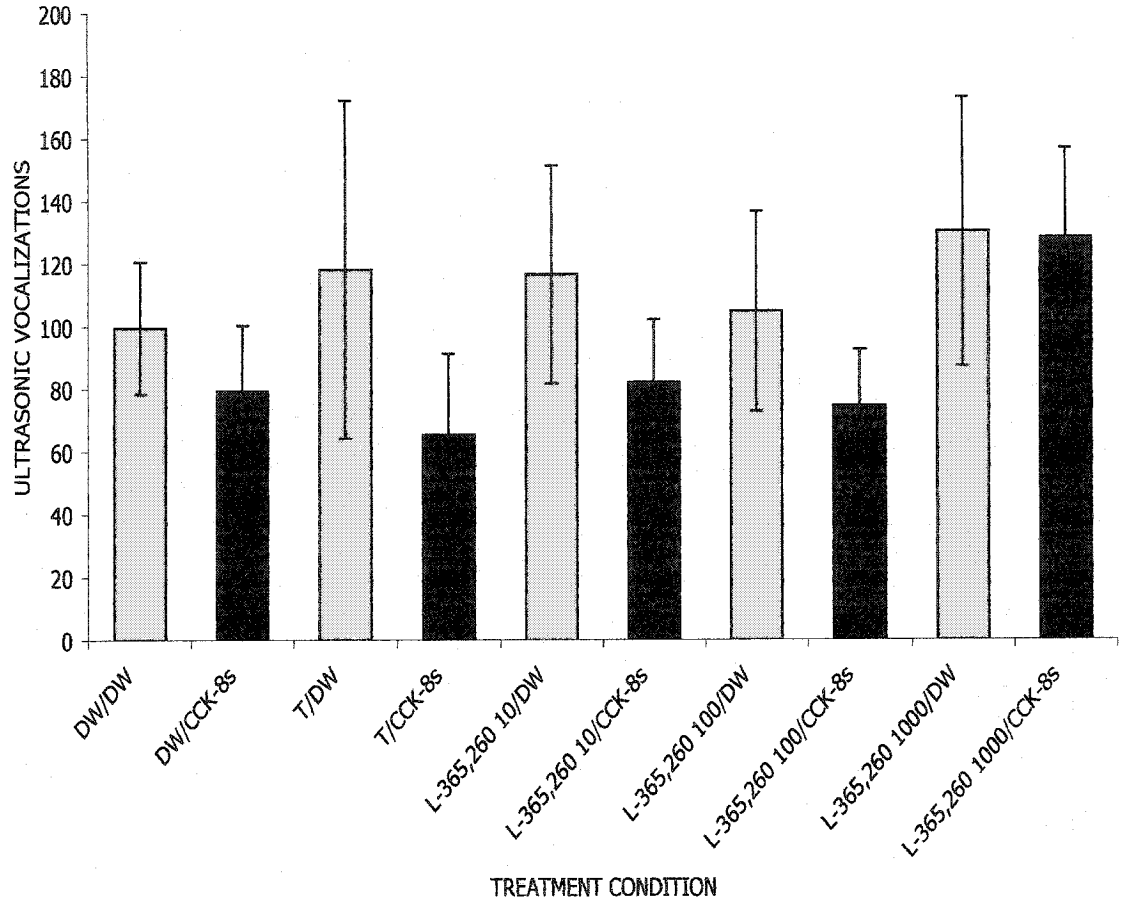
L-365,260 did not exert a significant effect on turning rates ($F_{4,79}$ = .177, p = .9499). Similarly, CCK-8s was without effect on Turns ($F_{1,79}$ = .360, p = .5501), and the interaction between L-365,260 and CCK-8s was not statistically significant ($F_{4,79}$ = .683, p = .6059; Figure 9c).

Rates of Head Raises were not altered by either L-365,260 ($F_{4,79}$ = .796, p = .5315) or CCK-8s ($F_{1,79}$ = 1.020, p = .3156). The interaction between L-365,260 and CCK-8s was also without effect ($F_{4,79}$ = .466, p = .7608; Figure 9d).

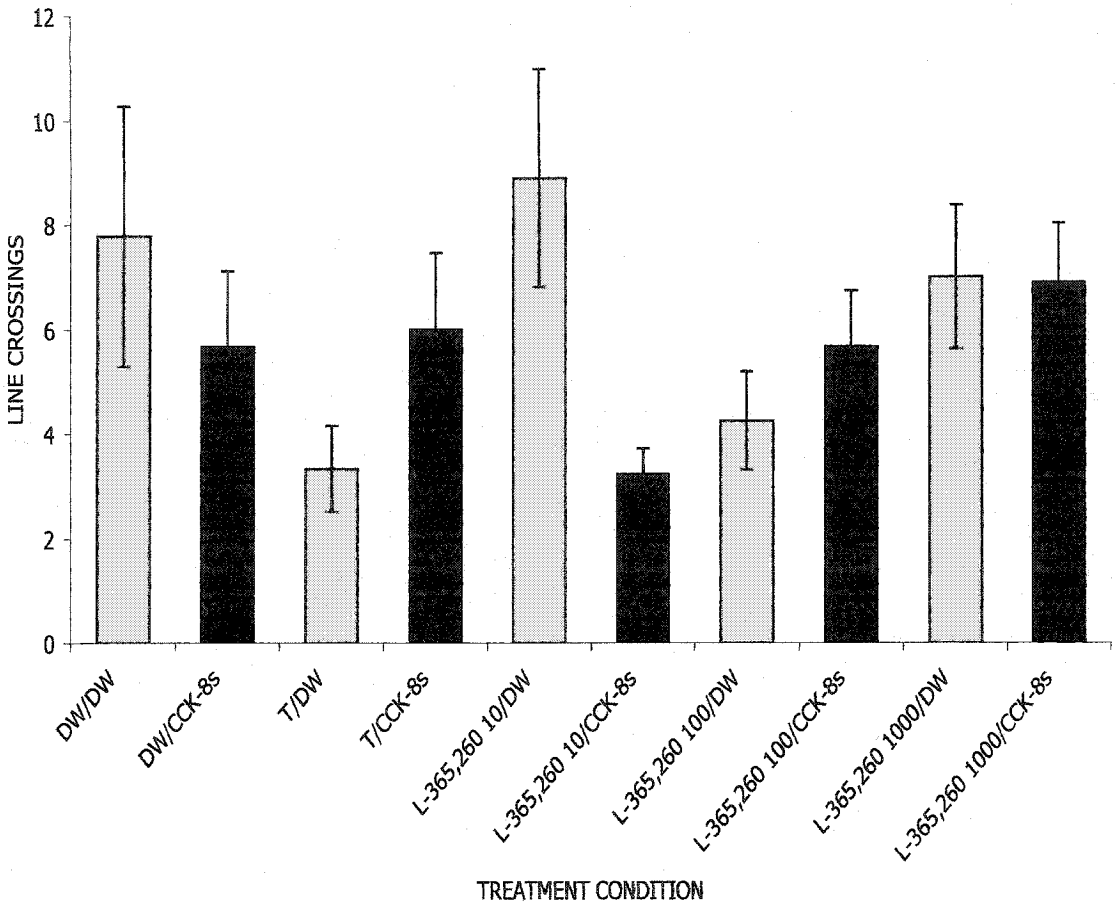
Finally, Wall Climbing was not significantly altered by L-365,260 ($F_{4,79}$ = .401, p = .8076), CCK-8s ($F_{1,79}$ = .760, p = .3860), or the interaction between these two compounds ($F_{4,79}$ = .098, p = .9828; Figure 9e).

3.3.2.3. Body Temperature Change

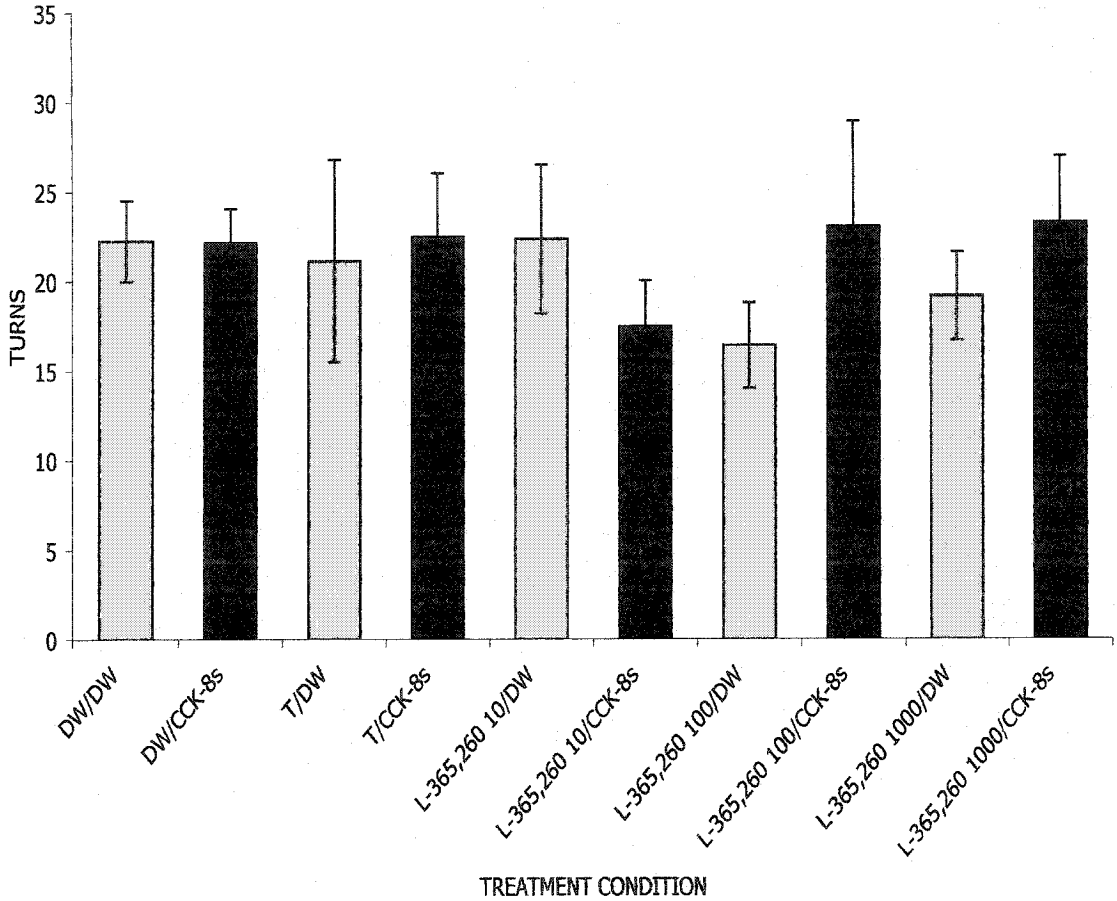
Following the conclusion of testing, there was no significant change in body temperature as a result of pre-treatment with L-365,260 ($F_{4,79}$ = .396, p = .8113) or CCK-8s ($F_{1,79}$ = 3.079, p = .0832). The interaction between the agonist and the antagonist also failed to alter subjects' body temperature to a significant degree ($F_{4,79}$ = .161, p = .9575; Figure 9f).



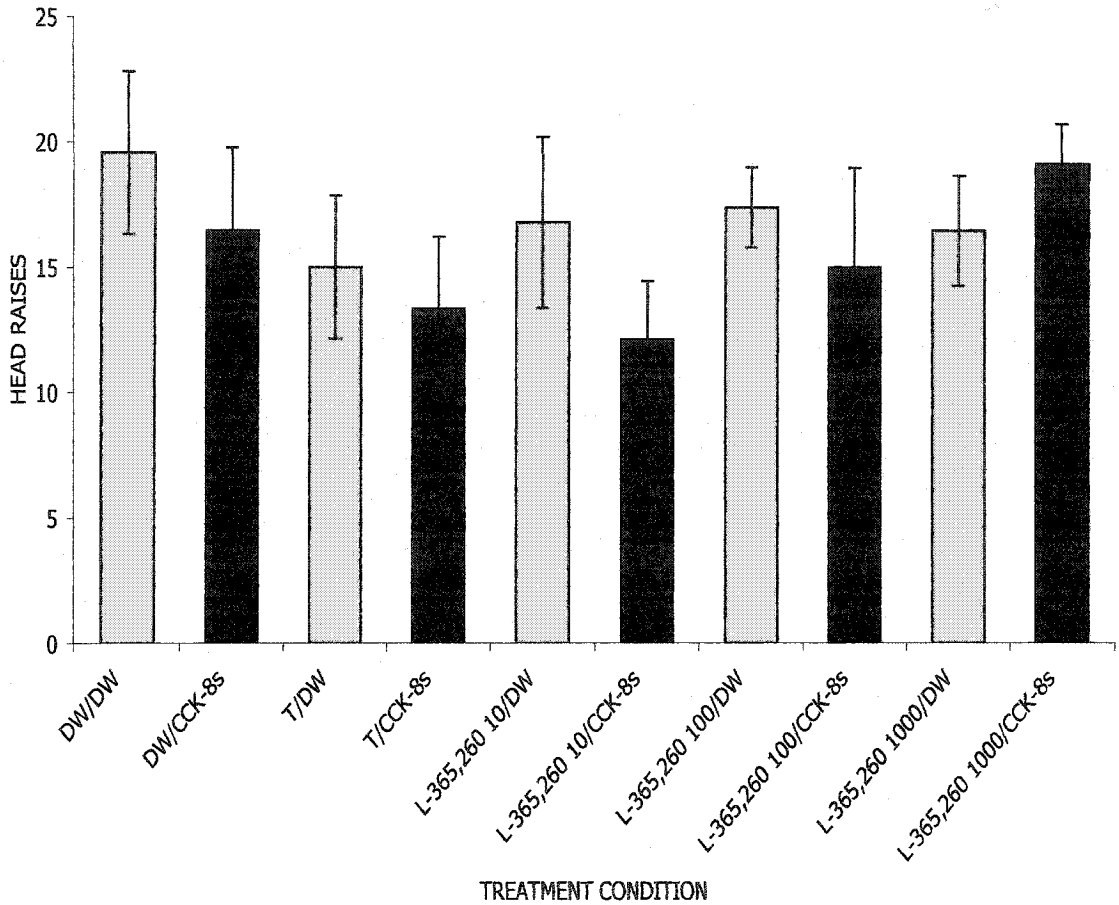
Figures 9a: Mean (±S.E.M.) UV rates across a 6-minute period, associated with the interaction between L-365,260 and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with L-365,260 (and 30 minutes following pre-treatment with CCK-8s).



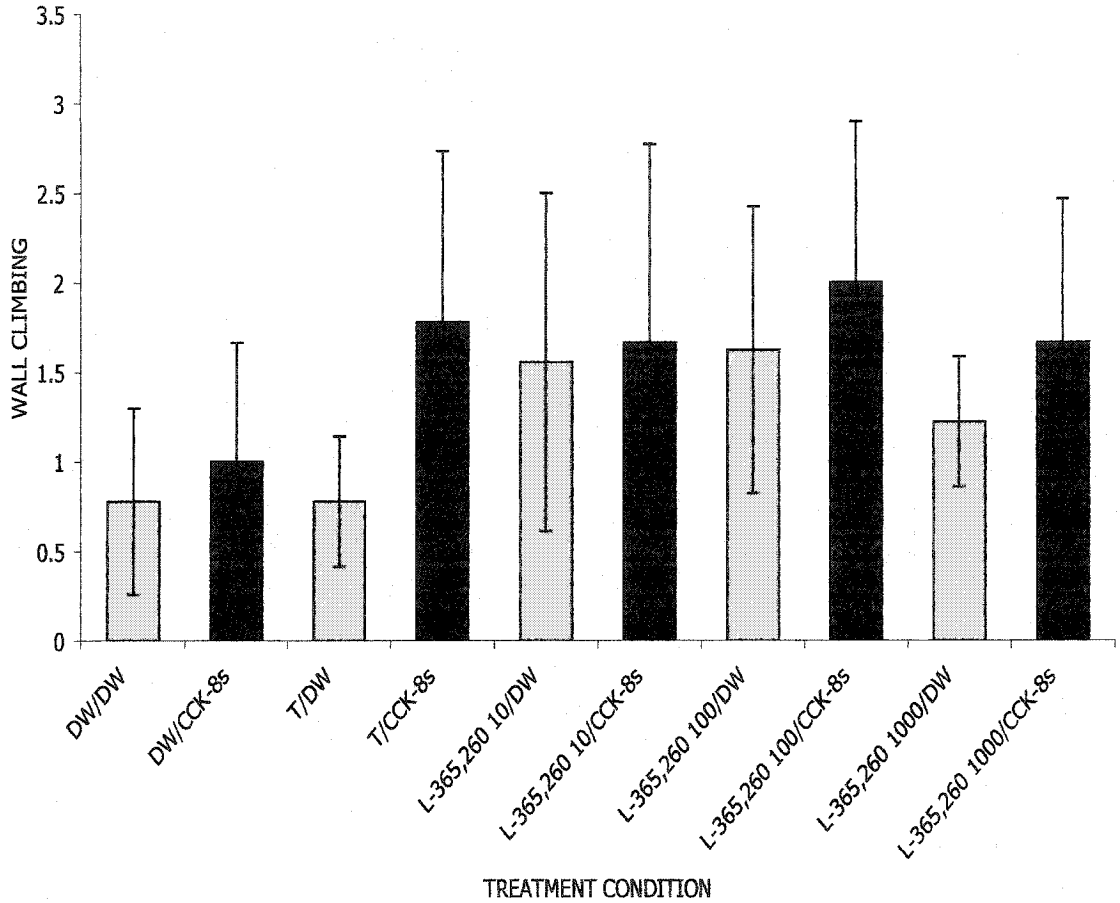
Figures 9b: Mean (±S.E.M.) rates of Line Crossing across a 6-minute period, associated with the interaction between L-365,260 and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with L-365,260 (and 30 minutes following pre-treatment with CCK-8s).



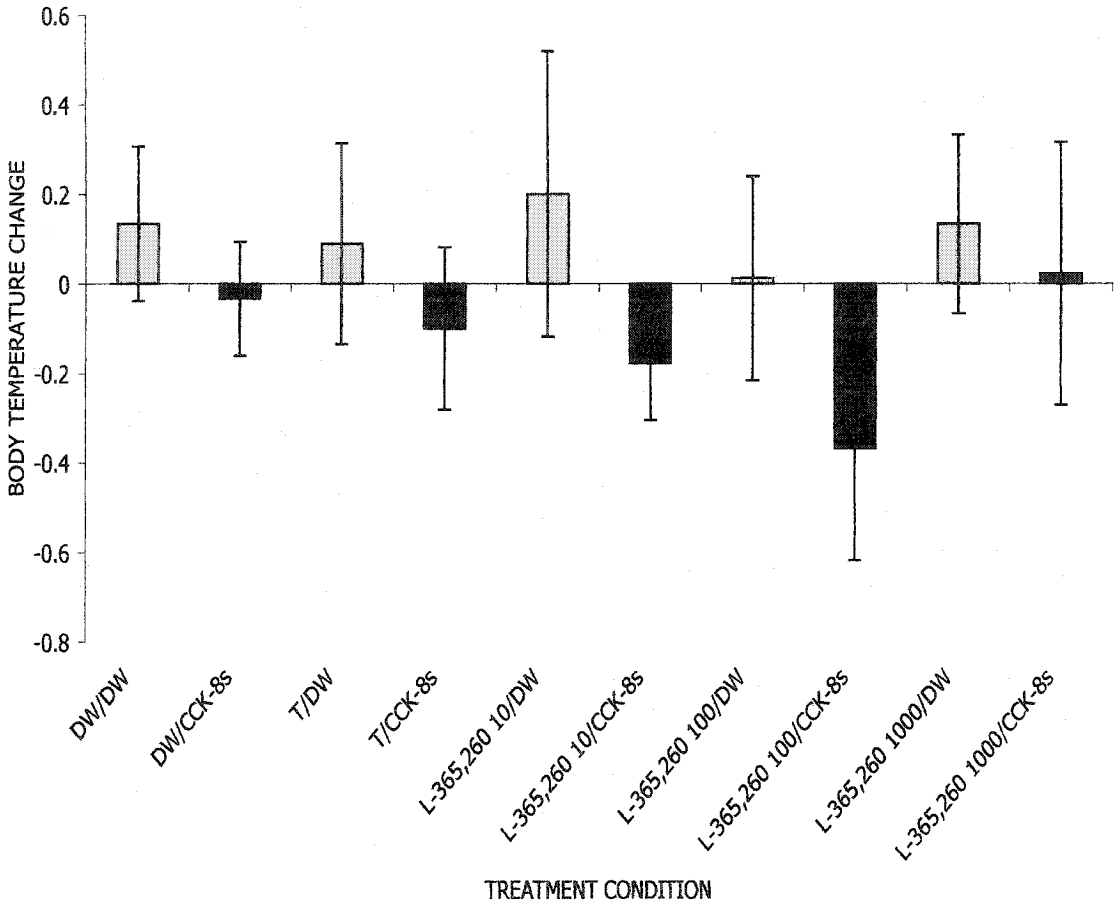
Figures 9c: Mean (±S.E.M.) Turn rates across a 6-minute period, associated with the interaction between L-365,260 and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with L-365,260 (and 30 minutes following pre-treatment with CCK-8s).



Figures 9d: Mean (±S.E.M.) rates of Head Raises across a 6-minute period, associated with the interaction between L-365,260 and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with L-365,260 (and 30 minutes following pre-treatment with CCK-8s).



Figures 9e: Mean (±S.E.M.) rates of Wall Climbing across a 6-minute period, associated with the interaction between L-365,260 and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with L-365,260 (and 30 minutes following pre-treatment with CCK-8s).



Figures 9f: Mean (±S.E.M.) Body Temperature Change across a 6-minute period, associated with the interaction between L-365,260 and CCK-8s. Behavioural testing was conducted 45 minutes following pre-treatment with L-365,260 (and 30 minutes following pre-treatment with CCK-8s).

3.4. Discussion: Experiments 5-6

Consistent with previous data (Blass & Shide, 1993; Weller & Dubson, 1998; Weller & Gispan, 2000), devazepide failed to induce any significant, intrinsic effects on isolation-induced UV rates. When examining the data associated with the main effect of devazepide (not shown), pups in the two control conditions (distilled water and Tween) emitted, on average, 86.5 and 93.9, calls over the 6-minute test session, respectively. Pups in each of the three devazepide conditions vocalized more frequently, with the highest rate of UVs noted in the 100 μg/kg treatment condition (122.2). The dose range employed in the current experiment (i.e., at, or below, 1000 μg/kg) was similar to that used in the above noted research. While it is possible that higher doses of devazepide might affect a change in isolation-induced UV rates, it is important to note that blockade of milk- or corn oil-induced reductions in UVs has been demonstrated with doses ranging from 300 μg/kg (Weller & Gispan, 2000) to 1000 μg/kg (Blass & Shide, 1993), indicating that the doses used in the current experiment are able to modify vocalization rates under some experimental conditions. Rather than attributing devazepide's failure to modify UVs to a dose-related issue, it is more likely the case that CCK-1 antagonism is effective in modulating UVs under some circumstances (i.e., after milk or corn oil intake), but not others (i.e., isolation). This interpretation is further reinforced by the finding that devazepide also had no effect on isolation-induced UVs when co-administered with CCK-8s (see below).

Devazepide also failed to exert any significant effects on the various indices of motor behaviour examined in the current experiment. This finding is in contrast to the increase in vertical activity (head raises and wall climbing), reported by Weller and Dubson (1998), following administration of 1000 μg/kg of devazepide in 6- to 9-day old Sprague-Dawley pups. Of note, the interval between drug administration and testing in the Weller and Dubson (1998) study was only 15 minutes, whereas in the current experiment, subjects were treated with the antagonist 45 minutes prior to testing. This difference in pre-treatment time may account for the discrepant findings. In this respect, inspection of the wall climbing and head raise (and line crossing) data in the current experiment reveals a trend towards increased behavioural activity following devazepide treatment. A briefer pre-treatment time might have resulted in significant main effects associated with these dependent measures. Age and/or strain differences between the two studies might also help explain the difference between the current results and those reported by Weller and Dubson (1998).

CCK-8s has previously been shown to induce hypothermia in adult rats (Crawley and Corwin, 1994), although in Experiments 3 and 4, CCK-8s did not alter body temperature significantly. As such, it is not surprising to find that blockade of CCK-1 receptors with devazepide was also without effect on body temperature. In this respect, in Experiment 5 there was a tendency for devazepide to decrease body temperature, although this effect was not statistically significant. Of note, other studies that have examined the effects of

devazepide on UVs (Blass & Shide, 1993; Weller & Dubson, 1998) have not included body temperature data, and so it is not possible to make inter-study comparisons regarding the effects of devazepide on this variable. Even if devazepide did significantly affect body temperature in these other studies, it is important to note that UV rates were not altered.

The CCK-2 receptor antagonist L-365,260 also did not affect UV rates to a significant degree. These data are the first to examine the effects of CCK-2 antagonism on isolation-induced UVs. While a potential anxiolytic effect might have been expected, given the fact that Rex et al. (1994) reported marked increases in UV rates following treatment with BOC-CCK-4, it is important to note that in the present series of experiments BOC-CCK-4 did not increase UVs beyond control group levels (see above). Accordingly, within the parameters of the current experiments it would appear that the CCK-2 receptor is not involved in the modulation of separation-induced UVs to any significant degree. Antagonism of the CCK-2 receptor also did not exert any significant main effects on indices of motor activity or body temperature.

In Experiment 4, CCK-8s reduced UV rates when administered 30 minutes prior to behavioural testing. This effect was significant across the entire dose range employed, and was specific in that the peptide did not affect motor activity or body temperature. However, CCK-8s did not reduce UVs in Experiment 5 or 6, as evidenced by the lack of a significant main effect in both experiments. In Experiment 5, UV rates were essentially identical between vehicle- and CCK-8s-

treated subjects (101.9 and 98.3, respectively). Although the difference between the two groups was more pronounced in Experiment 6 (113.9 for control subjects and 85.8 for CCK-8s-treated pups), this difference was not statistically significant.

The major difference between the procedures across these experiments is that in Experiment 4, pups received a single injection, whereas in the antagonist experiments, all pups were injected on two occasions (with vehicle or antagonist 45 minutes before testing, and then with vehicle or CCK-8s 30 minutes before testing). It is possible that the added stress associated with the additional injection in the antagonist experiments raised the pup's level of anxiety to the point where CCK-8s was unable to reduce call rates. However, if this were the case, one would expect higher rates of calling in the antagonist experiments, compared to those obtained in Experiment 4. However, in Experiment 4, the mean UV rate for the control group was 220, which is essentially twice as high as the rates associated with the control groups from the antagonist experiments (see above). Accordingly, it would appear that pups in the antagonist experiments were not more anxious as a result of being injected and handled twice. In fact, the opposite appeared to be the case. Perhaps the first injection in the antagonist experiments desensitized subjects to the stressful effects of the second injection, thus altering reactivity to CCK-8s and the subsequent isolation session, and resulting in an overall decrease in call rates in the antagonist experiments.

Another possible explanation for the lack of effect associated with CCK-8s in the antagonist experiments might be that pre-treatment with the antagonists altered the pups' behavioural response to CCK-8s. However, this explanation is unlikely given that the interaction effect for UVs was not significant in either experiment. In other words, CCK-8s did not affect UVs in pups that had previously been treated with one of the two vehicle substances (distilled water or Tween). The fact that two vehicle substances were used in each experiment also suggests that it is unlikely that the inability to replicate the anxiolytic effect of CCK-8s was due to some sort of physiological effect resulting from prior treatment with either one of the particular vehicle substances.

Although neither devazepide nor L-365,260 exerted significant main effects on motor activity, significant interaction effects were noted between each of the antagonists and CCK-8s. In the case of devazepide, pups treated with the highest dose of the CCK-1 receptor antagonist (1000 μg/kg) and then CCK-8s had lower rates of wall climbing compared to devazepide/vehicle treated subjects. This dose of devazepide also induced a significant increase in wall climbing in the Weller and Dubson (1998) study (as noted above, there was a trend towards a main effect for devazepide in the current experiment, this effect was not significant). Weller & Dubson (1998) postulated that endogenous CCK served to "calm" pups and pre-treatment with devazepide blocked this effect; essentially treatment with devazepide resulted in a behaviourally specific manifestation of heightened excitability. Such an interpretation is consistent with

the current finding that co-administration of CCK-8s resulted in lower wall climbing scores in devazepide-treated subjects. In Experiment 6, co-administration of L-365,260 (10 μg/kg) and CCK-8s resulted in lower line crossing rates compared to treatment with the CCK-2 antagonist and vehicle. This finding is difficult to interpret given that the lack of a similar interaction involving the lower or higher doses of L-365,260. In all likelihood, the significant interaction in this case represents a spurious finding.

Consistent with the lack of main effects for CCK-8s, devazepide, or L-365,260 on body temperature, there was no significant interaction between CCK-8s and either antagonist. Given that BOC-CCK-4 also did not alter body temperature, these data suggest that neither the CCK-1 or CCK-2 receptor is critically involved in thermoregulatory functions, as assessed under the current parameters.

4.0. GENERAL DISCUSSION

The data from the current set of experiments add to the inconsistent results that tend to dominate the literature regarding the effects of CCK fragments in animal models of anxiety. While the anxiolytic effect of CCK-8s, as detected in Experiment 4, is consistent with the findings of Weller and Blass (1988), it stands in contrast to the negative findings reported by Weller and Dubson (1998) and Rex and colleagues (1994). Indeed, variability regarding the effects of CCK-8s was even detected in the current study, as evidenced by the failure to replicate CCK-8s-induced anxiolysis (Experiment 4) in either of the

antagonist experiments (Experiments 5 and 6). Procedural differences could conceivably account for the variable findings across the above noted studies (and within the current set of experiments given the additional injection administered in the antagonist experiments). If such an interpretation were correct, it would seem that the effects of CCK-8s on isolation-induced UVs are far from robust.

The lack of any significant UV effects associated with BOC-CCK-4 in the current study calls into question the findings reported by Rex et al. (1994). As previously noted, Rex and colleagues did not include measurements of either body temperature or motor activity, and so it is not possible to determine whether BOC-CCK-4 exerted specific effects on UVs in their study. Clearly, the findings from Rex et al. need to be replicated in order to demonstrate that BOC-CCK-4 exerts anxioselective effects on isolation-induced UVs in younger rat pups.

Considering the findings with the CCK-1 receptor antagonist devazepide, the current data are consistent with the existing literature in demonstrating that blockade of CCK-1 receptors does not significantly impact UVs in isolated pups (Blass & Shide, 1993; Weller & Dubson, 1998; Weller & Gispan, 2000). In contrast, the data from Blass and Shide (1993) and Weller and Dubson (1998) indicate that UVs in response to other stimuli (e.g., milk and oil consumption) are indeed under the modulatory influence of CCK-1 receptors. The lack of an intrinsic devazepide effect on UVs is difficult to reconcile with the fact that CCK-1 receptor deficient rat pups exhibit significant elevated rates of isolation-induced

UVs (Weller et al., 2002), and further research is needed in order to fully understand the mechanisms underlying the results of their study.

The negative findings associated with the CCK-2 receptor antagonist L-365,260 are consistent with the negative BOC-CCK-4 data, and suggest that CCK-2 receptors are not critical in mediating UV responses to isolation (at least within the parameters of the current investigation). Whether or not CCK-2 receptors modulate UVs in response to other stimuli remains to be determined.

Given that the effects of CCK-8s were not replicated in either of the antagonist experiments, it would be necessary to replicate the findings from Experiment 4 (i.e., CCK-8s-induced anxiolysis associated 30 minutes following drug administration) in order to determine whether this effect represents a reliable finding. As matters stand, the overall pattern of results from the current experiments suggest that that manipulation of the CCK alone system does not result in reliable changes in isolation-induced UVs, or the very least, that CCK-8s-associated anxiolysis is present only under specific circumstances. Turning to the remaining dependent variables, drug-related changes pertaining to different indices of motor activity were limited to a single effect within each of the antagonist studies, and none of the compounds tested exerted any significant effects on body temperature.

The data regarding the role of CCK in the control of isolation-induced UVs in rat pups are currently unclear. While there would appear to be some evidence to suggest that CCK (mediated via the CCK-1 receptor) plays a role in modulating

UVs during isolation, too many inconsistencies exist in the literature to allow for any firm conclusions to be reached at this time. Assuming that exogenous CCK-8s administration does indeed induce an anxiolytic effect, this would appear to be limited to older pups (i.e., at least 11 days of age), and that other inter-study variables such as strain differences may influence the time-course of this effect.

The importance of strain/genetic differences in analyzing the effects of CCK in animal models of anxiety cannot be overemphasized. Significant strain differences have been noted with respect to UV rates in rat pups (Sales, 1979), and genetic breeding for rat pup UV rates (High vs. Low USV lines) has recently been demonstrated (Brunelli et al., 1997; Hofer, Shair, Masmela, & Brunelli, 2001). Finally, significant strain differences have been demonstrated for anxiety-related behaviours in adult rats (Onaivi, Maguire, Tsai, Davies, & Loew, 1992; Ramos, Berton, Mormede, & Chaouloff, 1997). Differences in strains tested (along with numerous other methodological differences) likely contribute to the inconsistent findings regarding the effects of CCK on rat pup UVs in particular, and in other animal models of anxiety.

One must also consider the possibility that the use of isolation-induced UVs does not represent a suitable model for studying the role that CCK plays in regulating anxiety-related behaviour. From a clinical perspective, distinct anxiety disorders exist, and the neuroanatomical and neurochemical substrates underlying these disorders are known to differ (Millan, 2003). As such, the term "animal model of anxiety" lacks a certain degree of specificity. The need for

refinement in the development of animal models of anxiety (i.e., to develop models with what could be termed as "disorder-specificity") is recognized amongst behavioural pharmacologists, and the notion that animal models of anxiety are interchangeable is outdated (Belzung, 2001; Bourin, 1997; Martin, 1998). Assuming that CCK dysfunction is particularly relevant to the study of panic, then one must consider whether isolation-induced UVs (or any other model) represent an appropriate choice as a model of anxiety/panic-like behaviour. Finally, it is important to note that the isolation-induced UV model of anxiety is the only widely used animal model of anxiety that involves the use of neonatal rats, and so developmental considerations should be taken into account when assessing the utility of this model for studying the effects of any given pharmacological compound. As previously noted, the example of clonidine is particularly relevant to this end; the compound has consistently been shown to increase UVs in pups (i.e., to be anxiogenic), although it possesses anxiolytic properties in adult rats and in humans. During the first two weeks of pup development (i.e., corresponding to the period when the vast majority of UV studies are conducted), the CCK system is in a state of constant development. As a result, the behavioural effects of CCK might vary depending on the precise age of the organism at the time of testing.

4.1. Future Directions

Future research regarding the putative effects of UVs on isolation-induced UVs should focus on attempting to resolve the discrepancies associated with data

from existing studies. A developmental study regarding the effects of BOC-CCK-4, CCK-8s, devazepide, and L-365,260 on UVs, motor activity, and body temperature would serve to clarify whether CCK plays a significant role in the manifestation of isolation-induced UVs during the postnatal period. As noted above, the effects of CCK-8s on UVs appear to be limited to older pups, whereas BOC-CCK-4 affected UVs only in younger pups. Given all the difficulties associated with making cross-study comparisons, a developmental analysis of these reported effects within one study (which includes the necessary measurements of motor activity and body temperature change to demonstrate specific effects on UVs) is warranted in order to determine whether manipulation of the CCK system results in age-dependent alterations in UVs. Subsequent use of CCK-1 and CCK-2 receptor antagonists would determine which of the CCK receptor subtypes is involved in mediating any observed effects on UVs.

The effects of CCK on UVs might vary as a result of strain differences. Although the existing data regarding the effects of CCK fragments on rat pup UVs involves the use of different strains (i.e., Sprague-Dawley, Wistar, and Long-Evans hooded), it is difficult to make direct strain comparisons across these data, owing to numerous other inter-study differences which might contribute to the discrepant UV findings. An evaluation of strain differences while controlling other procedural variables (e.g., duration of pre-treatment time, ambient temperature at testing, time of year of testing, etc.) would permit appropriate strain comparisons.

In addition, given that panic disorder patients exhibit a higher sensitivity to the panicogenic effects of CCK-4, it would be of interest to assess the effects of CCK fragments in "anxious" rats. In this respect, Podhorna and Brown (2000a,b) separated rat pups into "high-vocalizing" and "low-vocalizing" groups, based on the number of UVs emitted during a 1-minute baseline test session prior to drug treatment. A similar procedure might result in different UV effects following pre-treatment with CCK-based compounds. CCK might also exert different effects on UVs in the different N:NIH rat pup lines (see above) or in other rats that exhibit higher baseline levels of anxiety-related behaviour as a result of selective breeding efforts.

As the nosology of psychopathological conditions becomes increasingly complex, the methodology employed by researchers in the field of behavioural pharmacology will need to be refined. In particular, emphasis will need to be placed on developing and selecting the most appropriate animal model when attempting to study the neuropharmacological basis of a particular anxiety disorder. Assuming that CCK dysfunction is critically involved in human panic, the proposed lines of research, as outlined above, would help determine whether the study of isolation-induced UVs represents a useful animal model for studying panic-like behaviour in infrahuman subjects.

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