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Carl B. Schreck

The present study investigated the neurocrine and neuroendocrine control of locomotor activity, habitat choice, social behavior, and migratory behavior in juvenile chinook salmon (*Oncorhynchus tshawytscha*). This was achieved by the manipulation of three neurotransmitter systems; serotonin (5-HT), dopamine (DA), γ-amino-n-butyric acid (GABA) and the neuropeptide corticotropin-releasing hormone (CRH). Chemicals were administered into the third ventricle of the brain and both behavioral and physiological assays were used to evaluate the effects.

These studies established that CRH causes a dose dependent increase in locomotor activity. Interactions between CRH and the serotonergic system were also investigated. Activation of the serotonergic system potentiated the effect of

CRH on locomotor activity and the location of fish in the tanks. In addition, the role of GABA in the downregulation locomotor activity that was stimulated by CRH or by the combination of CRH and serotonin was investigated. The results suggest that endogenous GABA inhibits locomotor activity due to the interaction between CRH and serotonin but not CRH alone. By itself however, muscimol stimulated activity, an effect appears to be mediated by an indirect action on the dopaminergic system.

The present study also evaluated the effect of CRH on downstream swimming behavior in wild and hatchery juvenile chinook salmon. Fish were given an injection of CRH or saline and released into an artificial stream system. CRH increased the tendancy of nonmigratory fish to move downstream. However, in fish that were already migrating CRH significantly altered their behavior reminisient of the changes observed in field studies of stressed fish.

These results provide evidence that all four systems are involved in the regulation of locomotor activity. We hypothesize that CRH alters locomotor activity by stimulation of the serotonergic system and that this is inhibited by the action of GABA. In addition, we speculate that the dopaminergic mechanisms involved in the control of locomotor activity are indirectly mediated by GABA. This study also provides support for the hypothesis that CRH is involved in mediating some aspects of migratory behavior.

Central Control of Locomotor Activity in Juvenile Salmonids: The Role of Corticotropin Releasing Hormone in the Brain.

by

Shaun Clements

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Major Professor, representing Fisheries Science

Redacted for privacy

Head of Department of Fisheries and Wildlife

Redacted for privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Shaun Clements, Author

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CONTRIBUTION OF AUTHORS

Dr. Schreck provided laboratory facilities for the research and was involved in the research design and editing of each manuscript. Dr. Dickhoff and Dr. Larsen were involved in the editing of the 1st manuscript and Dr. Larsen performed the analysis of samples for thyroxine concentrations. Dr. Moore was involved in the research design and editing of the 2nd manuscript.

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Central Control of Locomotor Activity in Juvenile Salmonids: The Role of Corticotropin Releasing Hormone in the Brain.

INTRODUCTION

Background

Until 1980 the processes controlling and modifying locomotion in fish had received little attention. Since then much progress has been made in understanding the processes involved in the control of respiratory and cardiovascular systems in fish. In addition, the processes involved in exercise metabolism and recovery, with the exception of hormone effects, are now generally well known. In contrast, the neural control and generation of locomotory patterns is still poorly understood in teleosts.

The goal of this thesis is to further the understanding of the mechanisms promoting, sustaining, and modifying locomotion during stress. Such information may be especially valuable in cases such as on the Columbia River where salmon stocks are in steady decline. Based on field studies of fish behavior (Clugston and Schreck, 1992; Snelling and Schreck, 1993) it appears that the downstream migration of juvenile fish is modified by multiple stressors as the fish encounter a series of dams, making them more susceptible to predation, and reducing their ability to successfully complete the smoltification process. In addition, research in this area has implications for aquaculture and catch and release fisheries.

There is a large body of evidence to suggest that among mammalian vertebrates peptides and neurotransmitters of the central nervous system (CNS) can regulate

locomotor activity (Crine, 1981; Sutton et al. 1982; Moore et al. 1984). In particular, corticotropin-releasing hormone (CRH) is thought to play a major role in mediating stress related behaviors, including locomotor activity. CRH is a 41 amino acid peptide that was first isolated and later synthesized from ovine hypothalamic fragments that had CRH like activity (Vale, 1981). The synthetic peptide stimulated the release of adrenocorticotropic hormone (ACTH) and ß-endorphin from the adenohypophysis. CRH was first characterized in teleosts (*Catostomus commersoni*) by Morley et al. (1991). More recently CRH was also isolated and cloned from sockeye salmon (*Oncorhynchus nerka*) (Ando et al. 1999).

During the stress response the pituitary-interrenal system is activated via limbic inputs to the hypothalamic CRH system (Beaulieu et al. 1986). Given that the primary role of CRH appears to be stimulation of the release of ACTH, it is not surprising that a large proportion of the CRH and CRH immunoreactive cells and fibers within the CNS occur in the hypothalamic region. Recent evidence of extrahypothalamic CRH effects suggests that there might also be an alternative pathway by which physiological and behavioral stress responses may be mediated via direct neurotropic action of hormones in the CNS. CRH immunoreactivity has been identified in several extrahypothalamic structures, notably in the central nucleus of the amygdala, the olfactory bulb, the substantia innominata, the bed nucleus of the stria terminalis, and the region of the locus ceruleus and the parabranchial area (Battenberg et al. 1982; Swanson et al. 1983; Mancera &

Fernandez-Llebrez, 1995). The distribution of CRH perikarya and fibers among vertebrates appears homologous (reviewed by Peter, 1986). In addition, CRH like immunoreactivity appears consistent amongst vertebrates in the hypothalamic and extra-hypothalamic regions. In non-teleostean vertebrates, administration studies have shown that CRH can lead to electrophysiological (Siggins et al. 1985), neurochemical (Dunn and Berridge, 1987), autonomic (Brown et al. 1982), and behavioral (Sutton et al. 1982; Sherman and Kalin, 1987; Takahashi et al. 1989) responses.

Behavioral effects of CRH

When administered directly into the CNS, corticotropin-releasing hormone can affect behavior and enhance stress induced behavioral responses. Administration studies carried out on rats (Fisher and Sprague-Dawley) and rough-skinned newts (*Taricha granulosa*) indicate that CRH suppresses male sex behavior (Moore & Miller, 1984; Sirinathsinghji, 1987) and stimulates locomotor activity (Sutton et al. 1982; Moore et al. 1984). In addition, the response appears to be dose dependent. The increased activity observed in CRH treated animals persisted when the animals were hypophysectomised, but was not observed following systemic administration of CRH. Furthermore, pretreatment with dexamethasone, a corticosteroid feedback inhibitor, does not suppress the behavioral effects of exogenous CRH; these results suggest that CRH induced behavioral effects are mediated in the CNS via mechanisms independent of the HPI axis (Moore et al. 1984; Eaves et al. 1985;

Britten et al. 1986a,b). Locomotor activity was not blocked by the opiate antagonist naloxone or by dopamine receptor antagonists (Koob et al. 1984). In contrast, Saunders and Thornhill (1986) showed that naloxone, when given to rats 10 min prior to CRH, attenuated the stimulatory effect of CRH. Lowry et al. (1990) demonstrated that locomotor activity was enhanced in roughskinned newts when injected with naloxone 20 min prior to injections of CRH. This result suggests that handling of control animals prior to CRH injection stimulates the release of endogenous opiates that modulate the CRH effects on spontaneous locomotion. It appears then that the effect of opoids on CRH induced behavior is dependent on species.

It appears also that the environment is important in determining the CRH effects. The same intracerebroventricular (ICV) injections of CRH in rats produce behavioral activation in familiar environments, but resulted in reduced exploratory behavior in a novel, presumably more stressful, environment (Koob et al. 1993). Sutton et al. (1982) demonstrated a biphasic response to CRH injections in rats treated in novel, open environments. In contrast, other studies have found dose-dependent increases in locomotion in rats that were confined (Koob & Bloom, 1985). Few studies have investigated extrahypothalamic behavioral CRH effects in non-mammalian vertebrates. De Pedro et al. (1993) showed that CRH can increase food intake in goldfish (*Carassius auratus*) at high dosages (3.3 μg). Increased locomotor activity in rough-skinned newts has also been demonstrated following ICV CRH injection (Moore et al. 1984; Lowry et al. 1990).

Behavioral effects of a CRH antagonist

Although the results of studies using exogenously administered CRH have shown various behavioral effects, drawing conclusions about the physiological significance of such data is, at best, difficult given that the amount of CRH administered is generally far in excess of that available endogenously (it is estimated that the total hypothalamic content of CRH is 600-700 pg in the rat brain, (Fischman & Moldow et al. 1982, cited in Koob & Britton, 1990). However, CRH is a relatively large molecule and the site of action for such effects remains relatively unknown, it is possible that large concentrations of the CRH need to be injected if the molecule is to reach sites distant from the site of injection (Koob and Britton, 1990). To counter these arguments the role of endogenous CRH can be examined using an antagonist to CRH. In this way the effect of reducing endogenous activity can be observed. In rats the CRH receptor antagonist, α-helical CRH $_{(9\text{-}41)}$ (ahCRH) (Rivier et al. 1984), has been shown to suppress stress-induced neuroendocrine (Rivier et al. 1986) and autonomic (Brown et al. 1985) responses. In addition, recent research using ahCRH has provided support for the hypothesis that CRH plays a role in mediating stress induced behaviors (Krahn et al. 1986; Rivier et al. 1986; Berridge & Dunn, 1987; Tazi et al. 1987; Kalin et al. 1988; Winslow et al. 1989; Lowry & Moore, 1991; Heinrichs et al. 1992). Furthermore, ahCRH has also been implicated in blocking the activating and axiogenic actions of CRH in rats (Thatcher-Britton et al. 1986). Similarly, Cole et al. (1987)

demonstrated an attenuation of the conditioned fear response following administration of ahCRH to rats.

The results of these studies provide further evidence for the role of CRH in the behavioral response to stress. It appears that the CRH antagonist is particularly effective in mediating behavioral responses during stressful situations that involve psychological innervation. It is possible that ahCRH results in an "anxiolytic-like" effect. Alternatively, endogenous CRH may be involved specifically in the acquisition of conditioned fear (Koob & Britton, 1990)

The role of neurotransmitters in the control of locomotor activity.

CRH operates within a complex system of neurohormones and neurotransmitters, therefore interactions with other substances are likely to be important in determining behavioral output. Within the vertebrate brain the monoamines serotonin, dopamine and GABA appear to be particularly important in the control of locomotor activity. The concentration of serotonin (5-HT) within the brain correlates with changes in locomotor activity in goldfish (Fenwick, 1970) and the Texas killifish (*Fundulus grandis*) (Fingerman, 1976). Winberg et al. (1993) demonstrated that inhibition of brain serotonergic activity caused a significant increase in the activity levels of arctic charr (*Salvelinus alpinus*). In contrast, Genot et al. (1984) reported that inhibiting the synthesis of the serotonin precursor 5-HTP caused a significant decline in the activity of eels (*Anguilla anguilla*), and that the effect could be reversed by treatment with 5-HTP.

Less is known about the role of DA and GABA in regulating activity in fish. Mok and Monro (1998) reported that the addition of apomorphine (DA agonist) to the water significantly increased locomotor activity in tilapia (*Oreochromis niloticus* and *O. mossambicus*). Furthermore, this effect was abolished by the addition of DA antagonists to the water. In another study, injections of the dopamine agonist apomorphine resulted in significant decreases in both the time between successive wave peaks midbody (cycle period) and body curvature in adult sea lampreys (*Petromyzon marinus*) an effect that was opposed by 5-HT enhancement (Kemnitz et al. 1995). The behavioral effects of GABA in mammals appear to relate to both the dose and the site of injection (Tirelli, 1989). Plaznik et al. (1990) have reported that local injections of $GABA_A$ and $GABA_B$ agonists dose dependently decreased activity in rats. Similar results have been demonstrated following microinjection of muscimol into the posterior hypothalamus of domestic cats (Waldrop et al. 1988). In contrast, administration of the $GABA_A$ agonist muscimol into the medial septum or the ventral hippocampus increased locomotor activity in laboratory rats (Alvarez and Banzan, 1990; Osborne, 1994). There is some evidence that increases in locomotor activity in mammals following administration of GABA agonists can be attenuated by administration of dopamine (DA) antagonists (Osborne et al. 1993).

CRH and migration

The neural control or motivation for fish migration is poorly understood at present. A commonly sited example of fish migrations is the seaward journey of juvenile salmonids. During this migration the fish undergo a process that is described as the parr-smolt transformation or smoltification. At present CRH concentrations have not been measured during this transformation, however, plasma levels of cortisol are known to rise during this period (Specker & Schreck, 1982; Barton et al. 1986; Langhorne & Simpson, 1986). Given that CRH is involved in stimulating the release of ACTH, and thus cortisol, it is reasonable to assume that CRH levels are also elevated during this period. CRH may also be involved in the regulation of the hypothalamic-pituitary thyroid axis (Larsen et al. 1998). The HPT axis regulates many of the morphological and physiological changes during smoltification (Langdon, 1985). Based on these observations and the previously documented effect of CRH on locomotor activity in other vertebrates, I hypothesize that CRH may play a role in initiating migrations and coordinating the neuroendocrine control of fish migrations. In addition, field studies of juvenile salmon migrations have shown that exposure to a stressor will disrupt the migration of fish that are already moving downriver. Given the central role of CRH in the stress response, I also hypothesised that CRH is involved in controlling the behavioral response to stressors during the downstream migration.

Neurobiological sites for the behavioral actions of CRH

CRH has stimulatory effects on locomotion when injected at several brain sites (Brown, 1986). The forebrain noradrenaline systems derived from the locus ceruleus have been implicated in some of the response suppressing effects of CRH (Valentino et al. 1983; Valentino & Wehby 1988; Cole & Koob, 1988). Suppression of food intake is thought to involve a component acting directly on the paraventricular nucleus (PVN) (Krahn et al. 1988), although it is not clear whether the effect is due to the projection of the PVN to the median eminance or to brainstem autonomic systems. A third hypothesized site of action is the central nucleus of the amygdala. According to Koob et al. (1993) the amygdala is "well situated to play a role in the response to stress mediated by CRH." Experiments by Grey (1990) showed that pathways of the amygdala participate in behavioral responses to stress. Further, Lee and Tsai (1989) showed that injection of CRH into this area altered locomotion and exploratory behavior in rats.

Conclusions

The widespread distribution of CRH in brain regions outside the areas of the HPI axis in addition to the ascribed actions of CRH and ahCRH provide a reasonable basis for hypothesizing that CRH might have further roles besides controlling the HPI axis, indeed it may also be responsible for simultaneously activating and coordinating neuroendocrine, autonomic circulatory, metabolic, and behavioral responses during stress. To date, however, all studies, with the exception

of De Pedro (1993), have examined the neurobiology of CRH in mammals and amphibians. A mechanism in fish that is sensitive to input from multiple systems would allow considerable variability in both the intensity and type of behavioral output.

Objectives and Organization of the Thesis

The goal of this study is to identify the neural mechanisms that are involved in the control of locomotor activity in juvenile salmonids. To achieve this chapters 2-6 address specific objectives that determine the effect of CRH, serotonin, GABA and dopamine on a number of behaviors.

This thesis is organized into seven chapters. Following the introductory chapter are five chapters outlining the results of the research. Chapter II evaluates the effects of central administration on locomotor activity, social behavior, and habitat choice in juvenile chinook salmon. The results suggest that CRH mediates an increase in activity that is most likely due to extra-hypothalamic effects. There appears to be no effect of CRH on social behavior. However, administration of the antagonist for CRH significantly increased the time taken for fish to find cover. Chapter III investigates whether the effects of CRH in Chapter I are due to the mediation of serotonergic activity by CRH. The results suggest that this is the case as a serotonin uptake inhibitor potentiated the effect of CRH on activity. Chapter IV examines the role of GABA in the central control of locomotor activity. In this study a $GABA_A$ agonist effectively increased locomotor activity, an effect that was

opposed by the $GABA_A$ antagonist. There was no evidence, however, that GABA inhibited the behavioral effects of CRH on locomotor activity. Chapter V investigates the mechanism for the effect of GABA on locomotor activity. Specifically, the role of dopamine in this system is evaluated. The effect of GABA on activity was attenuated by administration of a dopamine antagonist and potentiated by administration of a dopamine uptake inhibitor. Chapter VI tests the hypothesis that CRH is involved in the initiation of downstream migration in juvenile salmonids. Further tests were used to examine if CRH alters migratory behavior using both hatchery fish and fish that were collected from the wild. The results provide support for the hypothesis that CRH is involved in initiating the migratory urge. Furthermore, there is evidence that CRH may mediate changes in migration reminiscent of those seen in field studies of migrating fish that have been stressed.

Chapter II is presented in manuscript form according to the specifications of the journal *General and Comparative Endocrinology*. Chapter III is presented in manuscript form according to the specifications of the journal *Hormones and Behavior*. Chapter IV is presented in manuscript form according to the specifications of the journal *Fish Physiology and Biochemistry*. Chapter V is presented in manuscript form according to the specifications of the journal *Hormones and Behavior*. Chapter VI is presented in manuscript form according to the specifications of the journal *Journal of Fish Biology*. Chapter VII is a general discussion of the results of this study.

CHAPTER II

CENTRAL ADMINISTRATION OF CORTICOTROPIN-RELEASING HORMONE STIMULATES LOCOMOTOR ACTIVITY IN JUVENILE CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)[1]

Shaun Clements, Carl B. Schreck,

Donald A. Larsen* and Walton W. Dickhoff*

Oregon Cooperative Fish and Wildlife Research Unit[2], Department of Fisheries and Wildlife and U.S.G.S (for C.B.S), Oregon State University, Corvallis, OR 97331-3803, USA. *The Integrative Fish Biology Program, Northwest Fisheries Science Center, National Marine Fisheries Service, 2725 Montlake Boulevard East, Seattle Washington 98112

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Abstract

The present study evaluated the effect of corticotropin releasing hormone (CRH) on locomotor activity, habitat choice, and social behavior in juvenile spring chinook salmon (*Oncorhynchus tshawytscha*). An intracerebroventricular (ICV) injection of CRH caused a dose dependent increase in locomotor activity. The stimulatory effect of exogenous CRH on locomotor activity lasted for at least 24 h. Injection (ICV) of a peptide antagonist of CRH, ∝-helical CRH_{9-41} (ahCRH), prevented the increase in locomotor activity when administered concurrently with CRH. Furthermore, fish administered the antagonist alone had significantly lower locomotor activity levels compared to saline injected control fish. The effects of CRH are often dependent on the social context. However, no evidence was found that the presence of conspecifics during the testing procedure affected locomotor activity following ICV injections of CRH. Similarly, ICV injections of CRH or ahCRH did not have a significant effect on the mean time spent in contact with a conspecific. However, the position of fish in the tank was affected by the treatments. ICV injections of CRH significantly increased the amount of time fish spent near the center of the tank. ICV injections of ahCRH significantly increased the mean time taken for fish to find cover in the tank. The effect of CRH and ahCRH on locomotor activity was not related to changes in plasma cortisol or thyroxine. These results support the hypothesis that endogenous CRH within the central nervous system (CNS) is involved in the stimulation of locomotor activity in fish. Furthermore, CRH may also alter habitat choice in a novel environment.

Introduction

Fish are exposed to a number of situations that are potentially harmful, such as unfavorable environmental conditions (temperature, toxins, hypoxia) or predation. In such cases, the generation of the appropriate locomotor response is essential for survival. Despite this, the neuroendocrine control and generation of locomotor patterns are poorly understood in fish.

It is well recognized, particularly in mammals, that hormones of the adrenal axis can regulate locomotor activity (Crine, 1981; Sutton et al. 1982; Moore et al. 1984). In particular, corticotropin-releasing hormone (CRH) is thought to play a major role in mediating stress related behaviors, including locomotor activity. CRH is the initiating hormone in the response of the hypothalamic-pituitary-interrenal (HPI) axis to stress. It stimulates production of ACTH in the pituitary, thereby playing a role in the regulation of peripheral responses to stress. In addition, Larsen et al. (1998) have shown that CRH may also induce the secretion of thyrotropin (TSH) in coho salmon pituitary cells *in vitro* suggesting that CRH may be involved in co-coordinating the actions of the hypothalamic-pituitary-thyroid (HPT) axis. A number of experiments suggest that thyroid hormones within the central nervous system (CNS) may also affect locomotor behavior in fishes (Hoar et al. 1952; Hoar et al. 1955, Sage, 1968).

However, CRH neurons are also widely distributed in brain regions outside areas associated with the HPI or HPT axes (Peter, 1986). Furthermore, Chappell et al. (1986) reported increased CRH-like immunoreactivity in extrahypothalamic

regions following acute or chronic stress. Behavioral tests have shown that CRH increases spontaneous locomotor activity in both mammals (Sutton et al. 1982) and amphibians (Moore et al. 1984; Lowry et al. 1990) independently of the pituitary.

In addition to its effects on locomotor activity, CRH is known to have anxiogenic properties. The administration of CRH in male rats caused a decrease in total interaction time, an increase in self grooming (To et al. 1999), and decreased exploratory behavior (Butler et al. 1990) consistent with increased anxiety. Furthermore, administration of the CRH peptide antagonist ahCRH significantly decreased non-social behaviors in male rats (Aloisi et al. 1999) and caused a dose dependent attenuation of the response suppressing effects of CRH in a conflict model of anxiety (Britton et al. 1986). The behavioral changes induced by CRH in mammals are reminiscent of those induced by stress. Furthermore, the effects of CRH are often dependent on the environment (Britton et al. 1982; Koob et al. 1993). Fish species encounter varied environments and often form aggregations for the purposes of feeding, migration and predator avoidance. Therefore, it may be hypothesized that the behavioral effects of CRH in juvenile chinook salmon change depending on the social context in which the fish was placed.

The widespread distribution of CRH in the brain together with its ascribed actions provide a reasonable basis for hypothesizing that CRH might have additional roles besides controlling the HPI axis in fish. Indeed, it may also be responsible for simultaneously activating and coordinating endocrine and behavioral responses to stress. The goal of this study is to determine if CRH has

activating effects on locomotor activity in fish. Secondarily, whether the behavioral effects of exogenous CRH are dependent on the social context, and whether such effects are consistent with an anxiogenic role for CRH in teleosts were investigated. To determine if CRH acted within the CNS, measurements of plasma cortisol and thyroxine were used as an indicator of activation of the HPI or HPT axes, respectively.

General Methods.

Fish

Juvenile spring chinook salmon parr (*Oncorhynchus tshawytscha*) (Willamette stock), were held under ambient photoperiod in a 336 L circular tank at Oregon State University's Fish Performance and Genetics Laboratory. Flow through water (12°C) was supplied from an artesian well. Fish were fed twice daily with semi moist pellet (BioOregon™). The fish in experiment 1 were 8 months old and 100.9 ± 0.9 mm (mean ± 1 SEM) in length. The fish in experiment 2 were 11 months old and 99.8 ± 1.23 mm in length. The fish in experiment 3 were 8 months old and 124.4 ± 1.22 mm in length. The fish in experiment 4 were 13 months old and 146.2 ± 3.15 mm in length.

Chemicals

Ovine CRH and the CRH antagonist, alpha-helical CRH_{9-41} (ahCRH), were obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in teleost

Ringers solution (0.2 % $NaHCO_3$ in 0.6 % NaCl solution). At the beginning of the study, preparations of the appropriate concentrations were made by serial dilution of the stock in the Ringers solution. A fresh preparation was used on each day of testing. Both the stock solutions and the daily preparations were stored frozen (-20°C) when not in use.

Administration procedure

Animals were netted from the holding tank and placed in anaesthetic (50 mg L^{-1} tricaine methanesulfonate buffered with 125 mg L^{-1} $NaHCO_3$) until they lost equilibrium. Chemicals were administered freehand under a dissecting microscope using a 10 µL glass syringe with a 26 G needle (Hamilton # 701). In preliminary trials this was found to be more accurate than using a stereotactic micrometer. Injections were performed midline, immediately behind the pineal gland. The syringe and needle were rinsed with Ringers solution between animals and were cleaned with ethanol and Ringers following each day's testing. The success of each injection was not checked during the experiments. However, in preliminary studies, injections with a dye tracer confirmed that the solution was being injected into the third ventricle, with > 85% accuracy. Furthermore, the variability in the response of the fish to each treatment was low, suggesting that in most instances the injection procedure was successful.

Behavioral tests

Locomotor activity was evaluated in an arena that consisted of a wooden tank, lined with dark green gelcoat. The inside dimensions were 150 cm x 31 cm x 21 cm (L x W x D). Ten identical arenas were used in this study. Well water (12°C) flowing at 7.5 L min^{-1} was introduced at the head of the tank and drained through a standpipe at the tail end. To minimize disturbance the arenas were surrounded by black polyethylene curtains. Each arena was lit by a 100 watt incandescent bulb mounted 1.2 m above the water surface. During the tests activity was monitored from above by 8 mm video cameras. During analysis the tank was divided into 8 equal segments by superimposing a grid onto the image. To compensate for parallax error the size of the segments was adjusted accordingly. Locomotor activity was quantified by counting the number of line crossings per unit time. Line crossings were recorded when the fish was actively swimming/gliding in a forward direction. Stereotyped circling behavior was not recorded as locomotor activity.

Social behavior, and habitat choice were evaluated in a separate arena. The inside dimensions were 96.5 cm x 96.5 cm x 60.9 cm. Two identical arenas were used. Each arena was filled with well water to a depth of 10 cm. The water was replaced every hour to maintain the temperature within 0.5 °C of the holding tanks. Each arena was lit by two 100 watt incandescent bulbs mounted 2.28 m above the water surface. Behavior was monitored by placing 8 mm video cameras directly above the arena. The cover in experiment 4 consisted of a 20 cm^2 opaque plastic

sheet placed in the center of the arena. The cover was mounted 4 cm above the bottom of the arena to allow fish to hide underneath.

Specific Methods.

Experiment 1

To determine if the injection procedure affected locomotor activity saline injected (2 μL) control fish were compared to fish that were anaesthetized but not injected. To determine if ICV CRH would increase locomotor activity, CRH was administered at 3 doses (500 ng, 1 μg, or 2 μg, in 2 μL Ringers solution). These doses were chosen following a review of the relevant literature, taking into account body size. The actual molar concentration ranges from 107 nmol to 428 nmol. However, it is likely that significantly less than the applied dose reaches the site of action due to leakage and loss by diffusion to other regions of the brain. To determine if basal and CRH induced locomotor activity is reduced by the CRH antagonist (ahCRH), fish were injected with either 1μg ahCRH (2 μL) (216 nmol) or 1μg CRH/1μg ahCRH (2 μL).

Following the injection fish were transferred to the testing arena and placed in a dark perforated plastic container for recovery. Twenty min later the container was removed and the fish was able to swim freely in the arena. Activity was recorded for a 10 min period beginning at the time of release. This length of time was chosen to maximise the opportunity to resolve treatment differences rather than differences due to random periods of burst swimming that could skew activity measures over a

shorter period. At the end of the recording period each fish was rapidly (< 1 min) netted from the arena and placed in a lethal dose of anaesthetic (200 mgL^{-1} Tricaine methanesulfonate buffered with 500 mgL^{-1} $NaHCO_3$). Each fish was measured (length, weight). A mixed arteriovenous sample of blood was collected in a pre-heparinized capillary tube by severing the caudal fin. The blood sample was centrifuged and the resultant plasma was drawn off and stored frozen (-80^{0}C) until it could be assayed for cortisol and thyroxine. Although blood was collected from all fish only the samples collected from fish treated with CRH 2 μg, ahCRH 1 μg, or saline were assayed. The experiment was conducted over a 1 week period beginning 21 June, 1998.

Experiment 2

The timecourse of the CRH-induced increase in locomotor activity and the effect of social context on locomotor activity was determined. Four treatment groups were tested for locomotor activity: ICV injection of saline (2 μL) and testing with or without conspecifics present; and ICV injection of CRH (1μg/2μL) and testing with or without conspecifics present. The experiment was conducted over 19 days, beginning September 8, 1998. Four fish were tested on each day, with two fish tested between 0900 and 1300 and two fish tested between 1400 and 1800. Only one treated fish (CRH or sham control) was tested at a time in the arena. The conspecifics (n = 8 in each trial) used in two of the treatment groups were held in separate arenas and transferred to the testing arena 10 min prior to the

introduction of the test fish. Following administration of the CRH or saline, the test fish was placed in a 20 L bucket and transferred to the testing arena. Behavior of the experimental fish was recorded for a 4 h period beginning as soon as the fish recovered equilibrium. The fish was then left for 20 h in the testing arena and a final recording of activity was made 24 h postinjection. Locomotor activity was later quantified by counting the number of line crossings made by the test fish in a 3 min period. Based on the results of experiment 1 it was decided that the low variability within treatments would allow a shorter recording period, and hence greater temporal resolution. For this experiment 3 min observations were made every 5 min starting at the time the fish recovered equilibrium up until 60 min post-recovery, then at 10 min intervals until 120 min post recovery, then at 180 min, 240 min and 24 h post recovery.

Experiment 3

To evaluate the effect of CRH on habitat choice/exploratory behavior and social behavior fish were injected ICV with either saline (1 μL), CRH (250 ng in 1 μL) or ah-CRH (1 μg in 1 μL). A preliminary trial indicated that 250 ng CRH was sufficient to cause increased locomotor activity in these fish. The fish were then held in a 20 L plastic container for 15 min with an untreated fish from the same home tank. Following this, both fish were released into the arena and their behavior was monitored for 10 min. During analysis the arena was divided into two areas of equal size. The outer area extended 55 mm from the 4 sides of the tank. Habitat

choice/exploratory behavior was evaluated by summing the total time the treated fish spent swimming in the inner area. Social behavior was evaluated by summing the total time the treated fish was within 20 cm (approx 2 body lengths) of the untreated fish. Locomotor activity was also measured by dividing the tank into 36 sectors and counting line crossings as in Expt 1. All behaviors were monitored during the first 10 min following release. Twenty animals were injected in each of the 3 treatments. The experiment was conducted during August of 2000.

Experiment 4

To evaluate the effect of CRH on the ability to find cover fish were injected ICV with either saline (1 μL) or ah-CRH (1 μg in 1 μL). The fish were then held in a 20 L plastic container for 25 min. Following this the fish was released into the arena through a 1 m PVC tube (10 cm diameter) and behavior was monitored for 20 min. The ability to find cover was quantified by measuring the time taken for the fish to reach the cover provided in the center of the arena. Twenty animals were injected in each treatment. The experiment was conducted during January of 2000.

Plasma Analysis

Plasma cortisol was measured by radioimmunoassay (RIA) following the method of Foster and Dunn, (1974) (modified by Redding et al. 1984). Plasma thyroxine was measured by RIA using the method developed by Dickhoff et al. (1982).

Statistical Analysis

Data for all treatments were normally distributed. However, there was unequal variance between treatments. Therefore, differences were analyzed using non-parametric procedures. Multiple sample comparisons in experiment 1 were performed using a Kruskal-Wallis test followed by a multiple range test (Dunns post-test). Only the following planned comparisons were considered during the post test: all treatments were compared to the saline controls; the dose effect of CRH (500 ng, 1 μg, and 2 μg) was analyzed; and, the effect of a concurrent injection of ahCRH and CRH was compared to ICV CRH alone. Differences between treatments in experiment 2 were analyzed by ANOVA with adjustment for serial sampling. Comparisons at specific timepoints were performed using the Mann-Whitney *U* test.

Results

Experiment 1

The injection procedure did not alter mean locomotor activity levels. There was no difference between uninjected fish and saline injected fish, therefore all further comparisons were made to the saline injected fish (Fig II.1). Intracerebroventricular injections of CRH induced a significant and dose-dependent increase in locomotor activity levels. The increase was highly significant ($P< 0.001$) at the highest concentration (CRH 2 μg) (Fig II.1). The increase in locomotor activity induced by CRH was blocked by the concurrent administration of the synthetic antagonist

(ahCRH). Furthermore, administration of the antagonist by itself resulted in a significant ($P < 0.05$) decrease in locomotor activity compared to saline injected fish (Fig II.1).

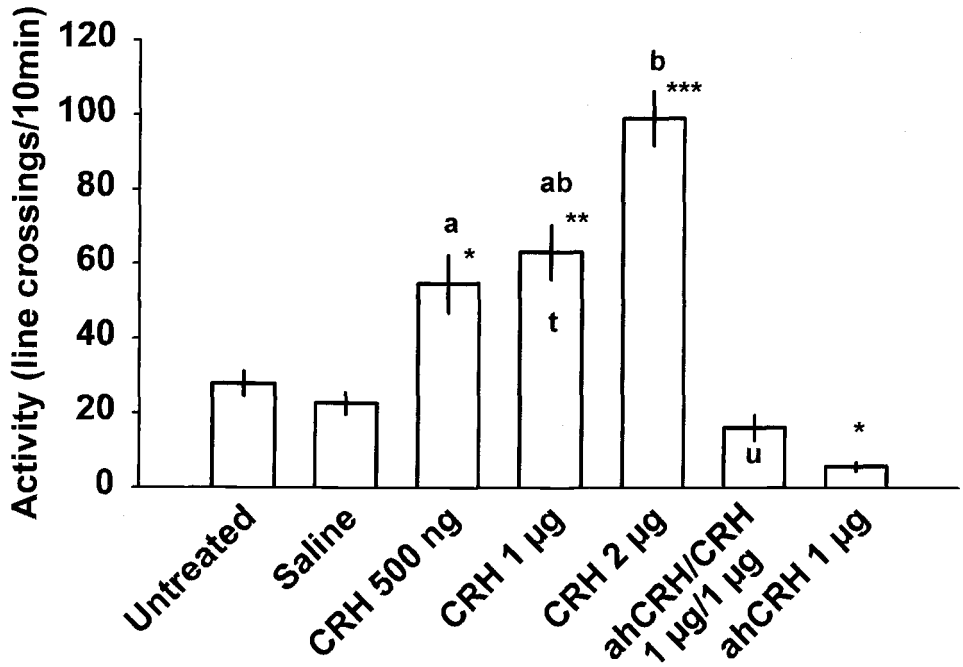

Figure II.1 Locomotor activity in juvenile chinook salmon following anaesthesia (untreated) or ICV injections of saline, CRH (500 ng, 1 µg, or 2 µg), a combination of CRH and ahCRH (1 µg CRH/ 1 µg ahCRH), or ahCRH (1 µg). Locomotor activity was quantified by placing the fish into a rectangular tank that was divided into 8 equal quadrants. Each column represents the mean (± 1 SEM) number of line crossings over a 10 min period starting 20 min post-injection ($N \geq 29$). Three separate analyses were performed on the data in this figure (see *Statistical Analyses* section of the methods for details). * Significantly different from saline control fish (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (all columns). Columns that share a common superscript are not different ($P > 0.05$) (columns 3, 4 and 5). Columns that share a common subscript are not different ($P > 0.05$) (columns 2, 4 and 6).

There were no differences in mean plasma cortisol concentrations between the fish that were treated with CRH 2 μg, ahCRH 1 μg, or saline (Fig II.2a). There was no difference in plasma T_4 concentrations between fish injected with saline or CRH at the highest dose (2 μg) (Fig II.2b). However, plasma T_4 concentrations were significantly ($P< 0.001$) higher in fish treated with the antagonist (ahCRH) compared to the CRH and saline treatments.

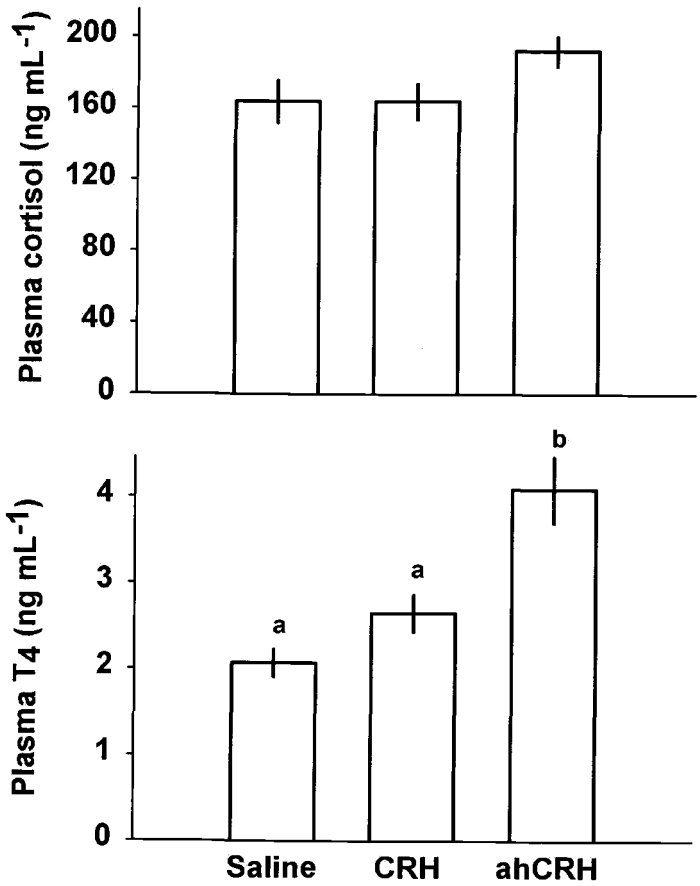

Figure II.2 Plasma cortisol (**a**) and thyroxine (**b**) concentrations in fish measured 26 min after ICV injection of saline, CRH (2 μg), or ahCRH (1 μg). Each column represents the mean ± 1 SEM of at least 19 fish. Columns that share a common superscript are not different ($P> 0.05$, Dunn's multiple range test).

Experiment 2

Locomotor activity levels of fish within a treatment did not change between days over the course of the experiment. Therefore data between days for all treatments were combined. For both treatment groups that were administered CRH mean locomotor activity levels were significantly elevated at all times compared to the appropriate control treatment. There were no differences between control or CRH treatments tested with or without conspecifics at any time (Fig II.3).

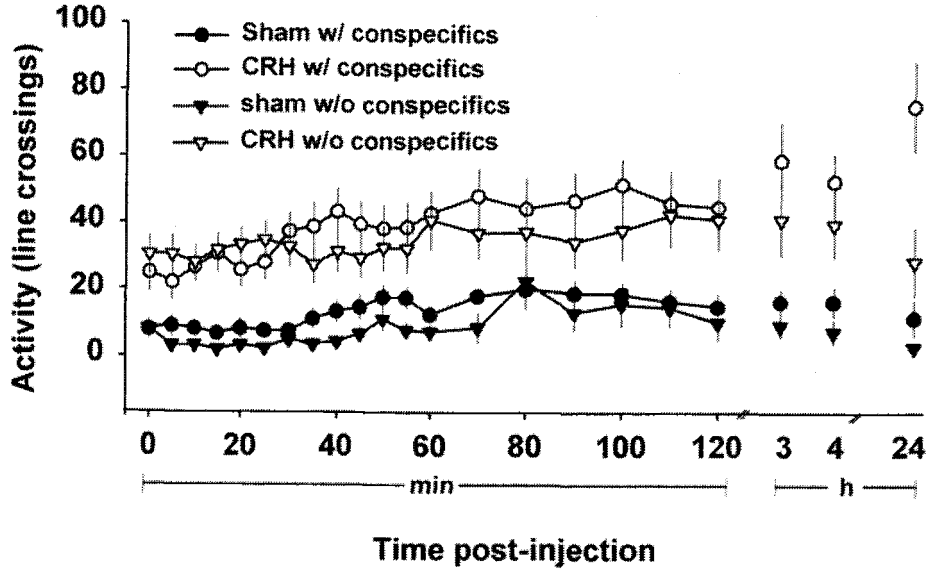

Figure II.3 Locomotor activity in juvenile chinook salmon following ICV injections of saline or CRH (500 ng) tested in the presence or absence of conspecifics. Locomotor activity was quantified by placing the fish into a rectangular tank that was divided into 8 equal quadrants. Each point represents the mean (± 1 SEM) number of line crossings over a 3 min period starting when the fishes recovered equilibrium. N = 20 for each treatment group. The groups receiving ICV CRH had significantly higher levels of locomotor activity relative to the appropriate control at all times ($P < 0.05$, Mann-Whitney U-test), except the CRH w/o conspecifics at 80 min. There were no differences in mean locomotor activity levels between the two groups receiving CRH or between the two groups receiving saline (ANOVA with adjustment for serial correlation).

Experiment 3

Fish that were given ICV CRH (250 ng) spent a significantly ($P < 0.05$) greater amount of time in the center of the arena compared to fish given either saline or ah-CRH (1 μg) (Table 1). There was no difference in the time spent in the center of the arena between fish given saline or ah-CRH (Table II.1). Both CRH and ah-CRH had no effect on the mean contact time between treated fish and untreated fish (Table 1). Locomotor activity was significantly elevated in fish given CRH ($P<0.001$). However there was no difference in locomotor activity between fish given ahCRH and saline (Table II.1).

Experiment 4

Ah-CRH (1 μg) significantly ($P < 0.001$) increased the amount of time taken for the fish to reach cover (666.05 ± 70.23s) compared to a sham injected fish (370.20 ± 79.45s).

Table II.1. The effect of ICV injections of CRH (250 ng) and ahCRH (1 μg) on the time spent within 2 bodylengths of a conspecific, the time spent in the center area of an open tank, and locomotor activity as measured by line crossings. Behavioral observations were made over a period of 10 min beginning 15 min after the injection. Each row represents the mean (1 SEM) of 20 fish. Treatments within a column that are not different share a common superscript (P> 0.05, Dunn's multiple range test).

Treatment	Time spent in proximity to conspecific (s)	Time in center area (s)	Locomotor activity (line crossings)
Saline	134.9 [a] (35.31)	69.5 [a] (27.9)	38.2 [a] (9.9)
CRH (250 ng)	71.5 [a] (13.9)	120.2 [b] (22.0)	148.5 [b] (19.1)
AhCRH (1 μg)	201.4 [a] (54.12)	46.8 [a] (14.4)	34.9 [a] (9.5)

Discussion

This study provides evidence that the administration of CRH into the 3rd ventricle induces a significant and dose dependent increase in locomotor activity in juvenile spring chinook salmon. Furthermore, the effect lasts over 24 h at a dose of 1 μg. The behavioral response to CRH demonstrated in this study is robust and repeatable, having been observed in 10 independent experiments over a 2 year period. Results showing that ahCRH can decrease locomotor activity compared to both saline and CRH treated groups are also consistent with the hypothesis that endogenous CRH is involved in the regulation of locomotor activity. This effect of CRH appears to be a general vertebrate response as previous research on mammals (Sutton et al. 1982) and amphibians (Moore et al. 1984) has also demonstrated that central administration of CRH can increase locomotor activity.

Previous work suggests that sociality is important among chinook salmon. Kelsey (1997) reported that juvenile chinook salmon normally show a high degree of shoaling behavior, evidenced, in part, by low inter-fish spacing, and very little aggression between fish. The administration of CRH is also known to alter social and exploratory behaviors in mammals (Kalivas et al. 1987; Winslow et al. 1989; Monnikes et al. 1992). These changes are generally thought of as anxiogenic and are often observed during the stress response. However, in the current study there was no evidence that CRH affects the amount of time juvenile chinook salmon spend in close proximity to a conspecific. This is in contrast to Dunn and File (1987) who reported that ICV CRH decreased the amount of time spent in social

interaction without altering locomotor activity in rats. There was also no evidence that the activating effect of CRH on locomotor activity was affected by the social context during testing.

Studies of fish behavior in the wild suggest that stress can alter habitat choice (Clugston and Schreck, 1992; Snelling and Schreck, 1993). The current study provides evidence that CRH alters exploratory behavior/habitat choice in juvenile chinook salmon, although whether this effect was independent of the stress response was not tested. Exogenous CRH significantly increased the amount of time fish spent in the center of the tank as opposed to the sides. Furthermore, this effect tended to be opposed when fish were given the CRH antagonist. It is not clear whether this represents an increase in exploratory behavior or if it is simply due to the higher probability of being in the center of the tank given the increased level of locomotor activity in these fish.

Similarly, Sigismondi and Weber (1988) demonstrated that stress significantly decreased the ability of juvenile chinook to find cover. Interestingly, the present results also show that the ability to find cover was significantly depressed when fish were given ahCRH. In addition, for both treatments the fish did not leave cover once they had found it. These observations may be interpreted in a number of ways; the increased hiding behavior may relate to the anxiogenic action of endogenous CRH, or to an increased exploratory behavior so the animal is more likely to encounter cover. The lack of established testing paradigms for emotionality in teleosts leads to difficulties in drawing conclusions about possible anxiogenic

properties of CRH. Therefore, further investigation is necessary to confirm that CRH has similar anxiogenic properties in teleosts to those reported in mammals. These results may provide evidence for a possible role for CRH in promoting predator avoidance by increasing the capacity of the fish to find cover.

A substantial body of evidence in mammals suggests that CRH alters locomotor activity and social behavior when injected into any of several brain regions (Brown, 1986). Furthermore, a number of researchers have demonstrated that the behavioral effects of CRH are mediated by direct neurotropic action in the CNS. Moore et al. (1984) reported that hypophysectomy did not attenuate the locomotor stimulating effect of CRH in rough skinned newts (*Taricha granulosa*) (Moore et al. 1984) nor was locomotor activity enhanced by injection of corticosterone or dexamethasone. Similarly, Britton (1986) showed that prior administration of dexamethasone did not attenuate locomotor activity or reverse the suppression of responding in the conflict test in rats. Several observations from this study support the hypothesis that the behavioral effects of CRH occur independently of the HPI axis. Measurements of plasma cortisol concentrations indicate that fish in representative treatments were stressed at the time of sampling. It was not possible to sample unstressed fish in this study due to the nature of the manipulations. However, basal levels for this stock of chinook salmon at this age and size have been established at the facility and range from 5 to 25 ng mL^{-1} (e.g. Barton et al. 1986; Barton and Schreck 1987). There were no differences in the magnitude of the plasma cortisol response between the treatments. Despite this, clear differences in locomotor behavior were

measured. In addition, the behavioral effects of CRH administration were observed immediately following recovery from anesthesia in the second experiment. In contrast, the cortisol response of the HPI axis generally peaks 30-60 min following exposure to a stressor in juvenile chinook salmon (Barton et al. 1986; Barton and Schreck, 1987). Based on the above evidence, it is unlikely that the fast-acting effect of CRH on behavior is mediated by the HPI axis.

Similarly, there were no significant differences in plasma levels of T_4 between fish given an injection of either saline or CRH (2 μg). Despite this, locomotor activity was significantly higher in fish administered CRH. Interestingly, however, fish treated with the antagonist had the highest levels of plasma T_4 and the lowest level of locomotor activity. This may be due to differences in the rate of conversion of T_4 to T_3 in the periphery, although such a hypothesis remains to be tested. These results suggest that, in the short term, increased thyroid activity is not causing the increased locomotor activity seen in fish administered CRH. However, a study using *in vitro* techniques suggests that ovine CRH can induce the secretion of TSH coho salmon pituitary cells (Larsen et al. 1998). Given that elevation of circulating thyroid hormones has been associated with increased activity in fish (Hoar et al. 1955) it is possible that the increased locomotor activity observed after several hours is due to CRH activating the HPT axis.

The present results provide evidence that CRH has activating effects on locomotor activity in fish, independently of the HPI and HPT axis. Furthermore, CRH alters habitat choice in an open environment. Involvement of CRH in the

activation of both central nervous system and the HPI axis in fish would be of adaptive value, allowing coordinated behavioral and physiological responses to stressful stimuli. Future work is needed to determine how such a system might function, and what effect it would have on behaviors such as schooling, migration, and predator avoidance.

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CHAPTER III

EVIDENCE THAT ACUTE BUT NOT CHRONIC SEROTONERGIC ACTIVATION POTENTIATES THE LOCOMOTOR STIMULATING EFFECTS OF CRH IN JUVENILE CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*).[1]

Shaun Clements, Frank L. Moore*, and Carl B. Schreck

Oregon Cooperative Fish and Wildlife Research Unit[2], Department of Fisheries and Wildlife, *Department of Zoology, and U.S.G.S (for C.B.S), Oregon State University, Corvallis, OR 97331-3803, USA

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Abstract

The present study investigated whether the serotonergic system is involved in mediating the behavioral effects of corticotropin-releasing hormone (CRH) in juvenile spring chinook salmon, *Oncorhynchus tshawytscha*. An intracerebroventricular (ICV) injection of CRH induced hyperactivity. The effect of CRH was potentiated by concurrent administration of the serotonin (5-HT) selective re-uptake inhibitor fluoxetine. However, administration of fluoxetine alone had no effect on activity. Conversely, ICV injections of the 5-HT_{1A} receptor antagonist NAN-190, attenuated the effect of CRH on locomotor activity when given in combination.

Chronic treatment with fluoxetine decreased locomotor activity and prevented the increase in activity caused by acute administration of CRH. Furthermore, chronic treatment with fluoxetine increased the amount of time fish spent near the center of the tank. Similar increases were seen in fish given chronic intraperitoneal (IP) injections of saline followed by an acute ICV injection of CRH. However the effect was not additive when fish were given chronic IP injections of fluoxetine followed by an acute ICV injection of CRH.

These results provide evidence to support the hypothesis that the effect of CRH on locomotor activity is mediated by the activity of the serotonergic system.

Introduction

Corticotropin-releasing hormone is the primary regulator of the hypothalamic-pituitary adrenal/interrenal axis in vertebrates. In addition CRH is involved in the regulation of many behavioral and physiological responses, particularly those involved with stress (Sutton et al. 1982; Moore et al. 1984; Dunn and Berridge, 1987). CRH has recently been characterized in sockeye salmon, *Oncorhynchus nerka*, by screening with PCR products amplified from the hypothalamic mRNA with primers deduced from the sequence of the sucker, *Catostomus commersoni,* CRH precursor (Ando et al. 1999). Furthermore, we have shown that intracerebroventricular (ICV) administration of CRH to juvenile chinook salmon, *Oncorhynchus tshawytscha*, can lead to behavioral changes such as increased activity (Clements et al. 2001b). However, CRH operates within a complex system of neurohormones and neurotransmitters, therefore interactions with other substances are likely to be important in determining behavioral output. Both *in vitro* and *in vivo* studies suggest that CRH is an important regulator of serotonergic activity. Lowry et al. (2000) showed that CRH increases the firing rates of a specific subpopulation of serotonergic neurons in the rat brain. Similarly, the administration of exogenous CRH can lead to the alteration of serotonin (5-HT) metabolism and neurotransmission (Singh et al. 1992). This functional link is further supported by topographical studies showing that serotonergic centers within the brain express CRH receptors, and are innervated by CRH fibers (Price et al. 1998).

The concentration of serotonin (5-HT) within the brain has also been correlated with changes in locomotor activity in goldfish, *Carassius auratus*, (Fenwick, 1970) and the Texas killifish, *Fundulus grandis* (Fingerman, 1976). Winberg et al. (1993) demonstrated that inhibition of brain serotonergic activity caused a significant increase in the activity levels of arctic charr, *Salvelinus alpinus*. In contrast Genot et al. (1984) reported that inhibiting the synthesis of the serotonin precursor 5-HTP caused a significant decline in the activity of eels, *Anguilla anguilla*, and that the effect could be reversed by treatment with 5-HTP.

Based on these findings we hypothesized that the increase in locomotor activity following administration of CRH was due to the activation of serotonergic mechanisms. Using behavioral and physiological assays we investigated whether the changes observed following CRH administration were affected by the up/down-regulation of serotonergic activity.

General Methods

Fish

Spring chinook salmon parr (8-10 months old, and 109.8 ± 1.4 mm in length) (Willamette stock), were held under ambient photoperiod in a 336 L circular tank at Oregon State University's Fish Performance and Genetics Laboratory. Flow through water (12^0C) was supplied from a well. Fish were fed twice daily with semi moist pellet (BioOregon™). All experiments were conducted between August and October 2000.

Chemicals

Ovine CRH, fluoxetine (5-HT selective re-uptake inhibitor) and NAN-190 (5-HT_{1A} receptor antagonist) were obtained from Sigma Chemical Co. (St. Louis, MO). Due to the insoluble nature of NAN-190 it was first dissolved in a 1:10 solution of DMSO then further diluted 1:10 in 1.1 X teleost Ringers solution (0.22 % $NaHCO_3$ in 0.66 % NaCl solution) to maintain the appropriate molality of the final solution. The remaining chemicals were dissolved in standard teleost Ringers solution (0.20 % $NaHCO_3$ in 0.6 % NaCl solution). At the beginning of each experiment aliquots of the appropriate concentrations were made by serial dilution of the stock in Ringers solution. A fresh aliquot was used on each day of testing. Both the stock solutions and aliquots were stored frozen (-20^{0}C) when not in use.

To test the effects of the DMSO vehicle on control behavior in experiment 4 a modified Ringers solution was made by diluting DMSO 1:10 in 1.1 X Ringers solution. In addition, CRH (500 ng) was dissolved in this modified Ringers solution to test the effect of DMSO on induced hyperactivity.

Administration procedure

Fish were netted from the holding tank and anaesthetized (5-7 min in 50 mg L^{-1} tricaine methanesulfonate buffered with 125 mg L^{-1} $NaHCO_3$). Chemicals were administered following a procedure described previously (Clements and Schreck, 2001). In summary, injections were performed midline, immediately behind the pineal gland into the 3rd ventricle. The needle was inserted to a depth sufficient to

penetrate the cartilaginous brain casing and enter the 3rd ventricle. The total injection volume was 1 μL. A preliminary trial established the accuracy of this procedure as approximately 85%.

Behavioral testing

Fish were injected and tested individually. The assignment of treatments and the arena for testing were random. Following the injection, each fish was transferred to the testing arena and placed in a dark perforated plastic container for recovery. Fifteen min later the container was removed and the fish was able to swim freely in the arena. The testing arena consisted of a light blue fiberglass tank with inside dimensions of 965 mm x 965 mm x 609 mm. Two identical arenas were used. Each arena was filled with well water to a depth of 10 cm. The water was replaced every hour to maintain the temperature within 0.5 °C of the holding tanks. Each arena was lit by two 100 watt incandescent bulbs mounted 2.28 m above the water surface. During the tests, activity was monitored from above by 8 mm video cameras for a 10 min period beginning at the time of release. During analysis of locomotor activity the tank was divided into 36 equal segments by superimposing a grid onto the recorded image. Activity was then quantified by counting the number of line crossings during the 10 min period. Line crossings were recorded when the fish was actively swimming/gliding in a forward direction. Stereotyped circling behavior was not recorded as activity. Habitat choice/exploratory behavior was evaluated by dividing the arena into an outer and inner area of equal size. The

perimeter of the inner area was 152 mm from each wall of the tank. The total time the fish spent swimming in the inner area was recorded.

Plasma Analysis

Plasma cortisol was measured by radioimmunoassay (RIA) following the method of Foster and Dunn, (1974) (modified by Redding et al. 1984).

Statistical Analysis

All data were analyzed using non-parametric methods because of unequal variances between treatments. Group differences were analyzed using a Kruskal-Wallis test. Treatment differences were analyzed using Dunn's multiple range post-test. Only planned comparisons were considered in the post tests. Differences between days within a treatment, and differences between the saline and CRH treatments and their respective DMSO controls in experiment 4 were analyzed using the Mann-Whitney *U* test.

Specific Methods and Results

Experiment 1: Dose response study: the effect of acute treatment with fluoxetine

To determine if enhancing endogenous serotonergic activity increases locomotor activity, fish were given an ICV injection of either saline or fluoxetine (10, 100, 1000 ng). Fifteen fish were injected in each treatment group. The experiment was conducted over two days. There was no difference in activity levels

within any of the treatment groups over the two days; therefore, we combined data within each treatment. ICV injections of fluoxetine had no effect on locomotor activity at any of the 3 doses (Fig. III.1).

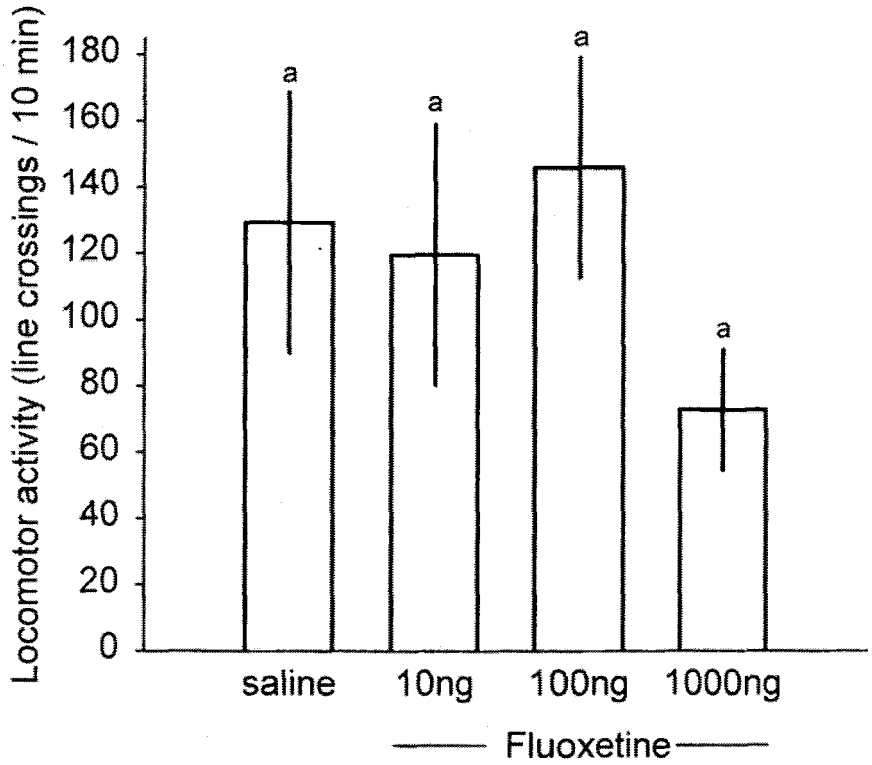

Figure III.1 Locomotor activity in juvenile chinook salmon following ICV injections of saline or fluoxetine (10, 100, 1000 ng). Locomotor activity was measured by placing fish in a square tank that was divided into 36 equal size quadrants. Each column represents the mean (± 1 SEM.) number of line crossings over a 10 min period starting 15 min post-injection. Columns that share a common superscript are not different ($P > 0.05$, Dunn's multiple range test). $N = 15$ for all treatments.

Experiment : Interaction between CRH and fluoxetine

To determine if the effect of CRH on locomotor activity was facilitated by serotonergic activity, fish were injected ICV with either saline, CRH (500 ng), or CRH (500 ng) and fluoxetine (10 or 100 ng) concurrently. Fifteen fish were injected in each treatment group. The experiment was conducted over 2 days. There were no differences in activity levels within any of the treatment groups over the two days; therefore, we combined data within each treatment. In agreement with previous results an ICV injection of CRH stimulated locomotor activity ($P < 0.05$) (Fig III.2). The concurrent administration of fluoxetine (100 ng) with CRH significantly potentiated the effect compared to CRH alone ($P < 0.001$).

Experiment 3: Dose response study: the effect of acute treatment with NAN-190

To further investigate the role of serotonergic activity in the control of locomotor activity, fish were injected ICV with either saline or the 5-HT_{1A} receptor antagonist NAN-190 (10, 100, 1000 ng). Fifteen fish were injected in each treatment group. The experiment was conducted on a single day. ICV injections of NAN-190 had no effect on locomotor activity at any of the 3 doses (Fig. III.3).

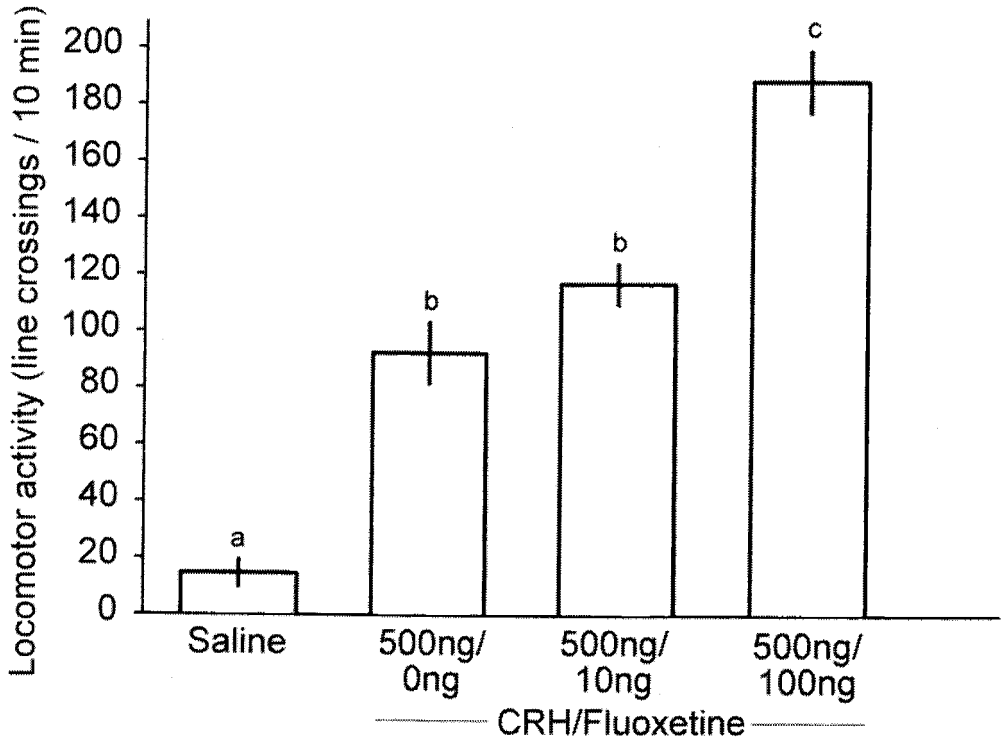

Figure III.2 Locomotor activity (mean ± 1 SEM) in juvenile chinook salmon following ICV injections of saline, CRH (500 ng) or a combination of CRH (500 ng) and fluoxetine (10 or 100 ng). Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($P > 0.05$, Dunn's multiple range test). $N = 15$ for all treatments.

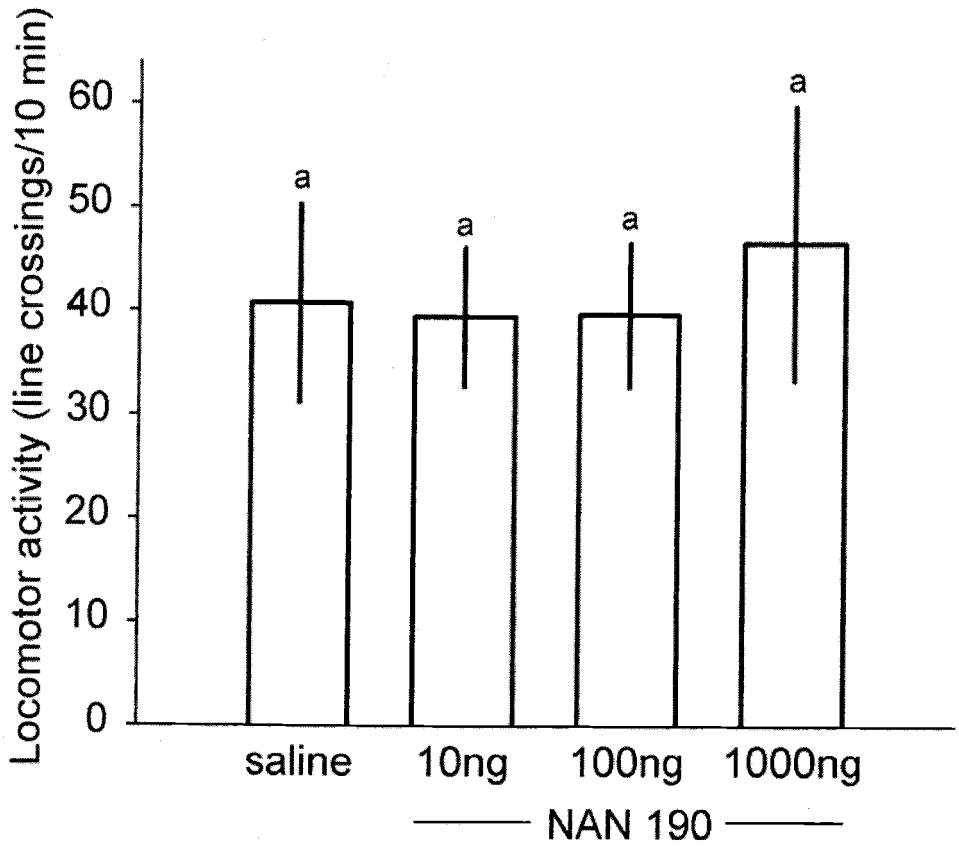

Figure III.3 Locomotor activity (mean ± 1 SEM) in juvenile chinook salmon following ICV injections of saline or NAN-190 (10, 100, 500 ng). Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($P > 0.05$, Dunn's multiple range test). $N = 15$ for all treatments.

Experiment 4: Interaction between CRH and NAN-190

To determine the effect of partially inhibiting serotonergic activity on CRH induced locomotor activity, fish were injected ICV with either saline, CRH (500 ng), or CRH (500 ng) and NAN-190 (10, 100 or 500 ng) concurrently. The effect of the DMSO vehicle on locomotor behavior was evaluated. Saline controls were compared to fish injected ICV with the modified Ringers solution containing DMSO. In addition, the effect of DMSO on induced hyperactivity was determined by comparing the treatment group given CRH dissolved in standard Ringers solution to a second group given CRH dissolved in the modified Ringers solution. Fifteen fish were injected in each treatment group. The experiment was conducted over 2 days. There was no difference in activity levels within any of the treatment groups over the two days, therefore we combined data in each treatment. DMSO had no effect on control or CRH-induced levels of activity. Therefore, all further comparisons were made to the original saline and CRH treatment groups that did not receive DMSO.

ICV injections of CRH stimulated locomotor activity ($P < 0.001$) (Fig III.4). The concurrent administration of NAN-190 with CRH tended to attenuate the effect of CRH alone, but the reduction was only significant at the highest dose (500 ng NAN-190) ($P < 0.001$).

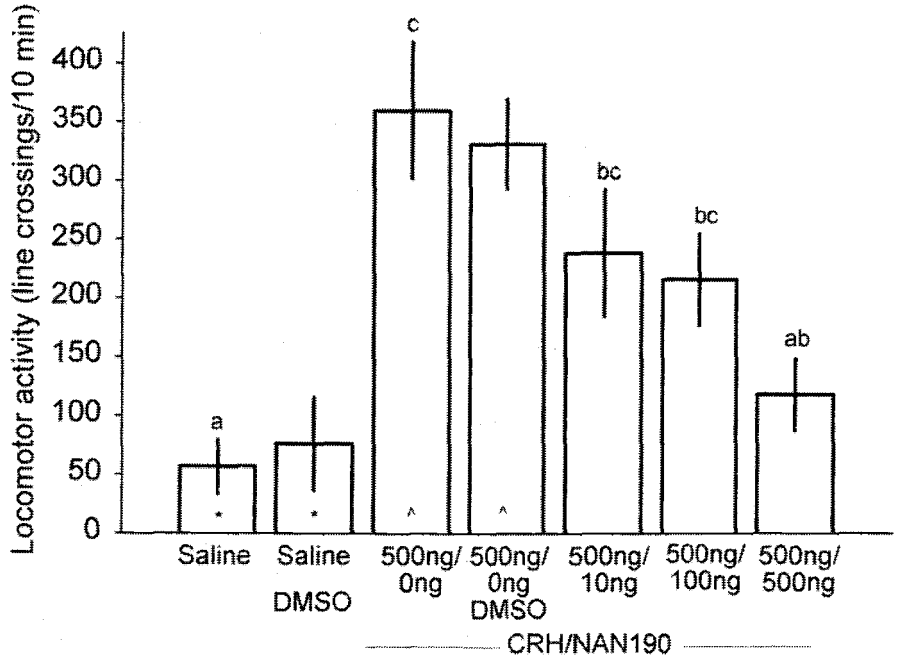

Figure III.4 Locomotor activity (mean ± 1 SEM) in juvenile chinook salmon following ICV injections of saline, a modified ringers solution containing DMSO (DMSO), CRH (500 ng), CRH dissolved in the modified ringers solution (CRH 500 ng/DMSO), or a combination of CRH (500 ng) and NAN-190 (10, 100 or 500 ng). Locomotor activity was measured as described in figure 1. Saline and CRH (500ng) were compared to their respective treatments with DMSO. Columns that share a common symbol are not different ($p > 0.05$, Mann-Whitney U test). Saline, CRH (500 ng) and CRH/NAN-190 (10, 100, 500 ng) were compared. Columns that share a common superscript are not different ($p > 0.05$, Dunn's multiple range test). $N = 15$ for all treatments

Experiment 5: Effect of chronic administration of fluoxetine on basal and CRH induced behaviors

To investigate whether chronic administration of fluoxetine would alter the behavior of fish in a novel environment fish were injected intraperitoneally (IP) with either saline or fluoxetine (2.5 mg/Kg) daily for 10 days then every second day for a further 10 days. The day after the final IP injection the two treatments were divided randomly with half the fish in each treatment receiving an ICV injection of saline and the other half an ICV injection of CRH (250 ng). Following the ICV injection the fish were placed in the behavioral testing arenas in a dark perforated plastic container for recovery. Fish behavior was recorded as described in the methods section. Only one fish was injected (ICV) and tested at a time. At the end of the recording period each fish was rapidly (< 10 s) netted from the arena and placed in a lethal dose of anaesthetic (200 mg L^{-1} tricaine methanesulfonate buffered with 500 mg L^{-1} $NaHCO_3$). Each fish was measured (length, weight). A mixed arteriovenous sample of blood was collected in a pre-heparinized capillary tube by severing the caudal fin. The blood sample was centrifuged and the resultant plasma was drawn off and stored frozen (-80^{0}C) until it could be assayed for cortisol.

Fish that were treated with saline (IP) followed by an ICV injection of saline had significantly higher levels of locomotor activity compared to fish that had only been given an ICV injection of saline ($P < 0.001$) (Fig III.5a). This effect was significantly attenuated by chronic (IP) treatment with fluoxetine ($P < 0.05$). ICV injections of CRH did not significantly change locomotor activity in either

treatment (chronic saline or fluoxetine) compared to fish that were given saline ICV.

Chronic IP injections of saline had no effect on habitat choice (Fig III.5b). In contrast, IP injections of fluoxetine significantly increased the amount of time individual fish spent in the center of the arena ($P < 0.05$). Habitat choice in fish that received an ICV injection of CRH following chronic IP injections of saline was not different from any of the treatments. In addition, the effect of chronic fluoxetine was not additive when fish were given fluoxetine (IP) and CRH (ICV).

There were no differences in plasma cortisol levels among the treatments that were given chronic IP injections (Table III.1).

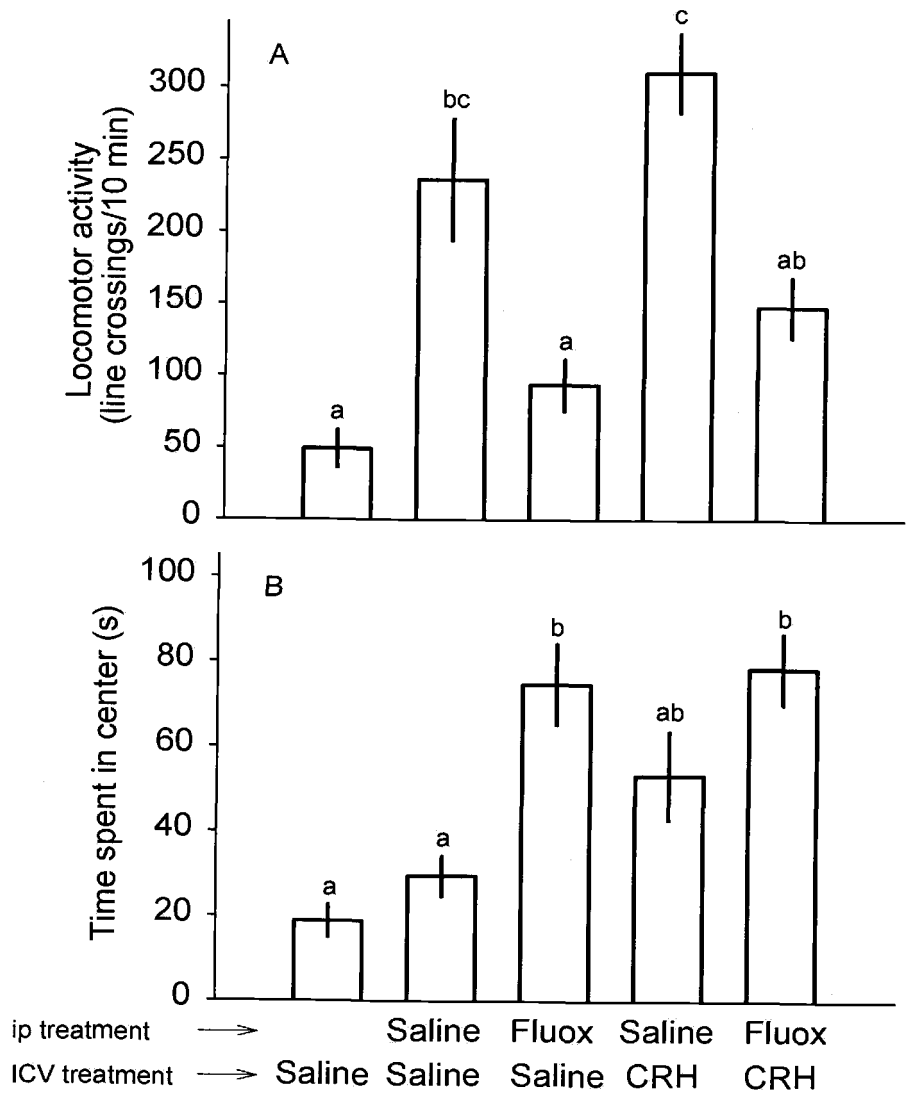

Figure III.5 Activity levels (5a) and habitat choice (5b) (mean ± 1 SEM) in juvenile spring chinook salmon following an ICV injection of: saline, chronic IP injections of saline followed by an ICV injection of saline or CRH (500 ng), or chronic IP injections of fluoxetine (2.5 mg/Kg) followed by an ICV injection of saline or CRH (500 ng). Locomotor activity was measured using a procedure outlined in Fig.III.1. Habitat choice was measured by dividing the tank into 2 areas of equal size, one inside of the other and summing the time spent in the middle of the tank. $N = 10$ for all treatments. Columns that are not significantly different share a common superscript ($P > 0.05$, Dunn's multiple range test).

Table III.1. Plasma levels of cortisol (mean ± 1 SEM (*N*)) after a 20 day course of IP injections with either saline or fluoxetine, followed by an ICV injection of either saline or CRH. There are no significant differences between treatments.

IP treatment	ICV treatment	Plasma cortisol
Saline	Saline	149.2 ± 10.6 (*9*)
Fluoxetine (2.5 mg/Kg)	Saline	130.0 ± 8.1 (*7*)
Saline	CRH (500 ng)	145.3 ± 7.0 (*9*)
Fluoxetine (2.5 mg/Kg)	CRH (500 ng)	122.1 ± 7.3 (*6*)

Discussion

Acute administration of fluoxetine into the CNS clearly potentiated the effect of exogenous CRH on locomotor activity. *In vitro* experiments suggest that serotonin stimulates the release of CRH (Jones et al. 1976; Tizabi and Calogero, 1992). Therefore, one hypothesis is that the increase in locomotor activity is due to the release of CRH following stimulation by serotonin. However, if this were the case injections of fluoxetine alone should cause an increase in locomotor activity. By itself fluoxetine had no significant effect on locomotor compared to control fish therefore, a more likely explanation is that the increased activity is due to the stimulation of the serotonergic system by CRH. Furthermore, this action may be mediated by the 5-HT_{1A} receptor. The involvement of the 5-HT_{1A} receptor in controlling locomotor activity has been proposed previously for mammals (Wedderburn and Sillar, 1994; O'Neill and Sanger, 1999), and may be due to stimulation of noradrenaline (Suwabe et al. 2000). In our study, injections of the 5-HT_{1A} antagonist, NAN-190 significantly attenuated CRH induced locomotor activity in juvenile chinook. Similarly, long term treatment with fluoxetine

attenuated the locomotor response compared to controls, and following administration of CRH ICV. This may be due to the depletion of 5-HT within the brain, however it may also be due to an effect on the 5-HT receptors. Previous studies have shown that chronic fluoxetine treatment results in the desensitization of the 5-HT_{1A} receptor (Berlin, et al. 1998; Raap, et al. 1999).

Investigations of the role of 5-HT in locomotor activity are often contradictory in vertebrates, with either excitatory or inhibitory effects being reported. Fingerman (1976) and Winberg et al. (1993) suggested that increased serotonergic activity is associated with reduced locomotor activity in fish. In contrast, our results and those of Genot (1984) suggest that serotonin can also facilitate increases in activity. This apparent contradiction may arise due to experimental differences in the manipulation of serotonergic activity. Depletion of serotonin within the CNS is often associated with increased levels of motor activity in mammals (Bradford, 1986), suggesting that serotonin is likely to be inhibitory at a macroscopic level with respect to locomotor behavior. Long-term treatment with fluoxetine tends to decrease levels of 5-HT in the brain (Caccia et al. 1992; Trouvin et al. 1993; Hall et al. 1995). However, we found no change in activity levels following chronic administration of fluoxetine. In contrast, increases in synaptic levels of serotonin, or stimulation of post-synaptic serotonin receptors can induce hyperactivity in rats and mice (Grahame-Smith, 1971). This is in agreement with our results that demonstrated that acute treatment with fluoxetine potentiated the effect of CRH.

Previously, we showed that exogenous CRH significantly increased the amount of time fish spent in the center of the tank as opposed to the sides. Furthermore, this effect tended to be opposed when fish were given the CRH antagonist. Similarly, in the current study chronic treatment with fluoxetine significantly increased the amount of time fish spent in the center of the tank. This could be interpreted as risk taking behavior as fish in the open are more likely to be taken by predators. Given that the effects of CRH and fluoxetine were not additive, it is possible that the same effect is achieved via different mechanisms. CRH tends to decrease exploratory behavior in rats (Butler et al. 1990; Spadaro et al. 1990), therefore apparent changes in positional behavior in these fish may be due simply to the higher probability of being in the center of the tank given the increased level of locomotor activity in these fish. In contrast, fish that were treated with fluoxetine had low levels of locomotor activity. The effect of fluoxetine on positional behavior may instead relate to its anxiolytic properties, whereby the psychological perception of a stressor is lowered. Tsuji (2000) suggested that the 5-HT_{1A} receptor is involved in the recognition of and the ability to cope with a stressor. If this is true then it would be interesting to determine whether the changes in positional behavior in the current study were due to desensitization of the 5-HT_{1A} receptor following chronic treatment with fluoxetine.

In previous studies, we were not able to correlate the behavioral changes to any changes in the endocrine response to stress (Clements et al. 2001b). In that study, fish from all treatments had high cortisol levels. This is most likely due to the

administration procedure itself. Similarly, in the current study long term treatment with fluoxetine had no effect on the stress response when measured 25 min after the final treatment. These findings are consistent with a study by Zhang et al. (2000) who found that long term fluoxetine treatment produced behavioral effects but did not inhibit the stress response.

Based on these results we speculate that the increases in CRH activity during stress (Rivier and Plotsky, 1986; Lederis et al. 1994; Chappell et al. 1996) are responsible, in part, for the changes in serotonergic activity observed following a stressor (Winberg et al. 1992; Winberg and Nilsson, 1993). This link was proposed by Lowry et al. (2000) and is supported by topographical (Cummings et al. 1983), behavioral (Lazosky and Britten, 1991), and physiological studies (Singh et al. 1992). In fish CRH and serotonin may regulate both locomotor behavior and habitat choice in response to a stressor. Such a system would allow for co-ordinated physiological and behavioral responses. In summary, the current study provides evidence to support the hypothesis that the stimulatory effect of CRH on locomotor activity is due, in part at least, to interactions with the serotonergic system.

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CHAPTER IV

THE $GABA_A$ AGONIST MUSCIMOL ENHANCES LOCOMOTOR ACTIVITY, BUT DOES NOT ALTER THE BEHAVIOURAL EFFECTS OF CRH IN JUVENILE SPRING CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)[1].

SHaun Clements and Carl B. Schreck

Oregon Cooperative Fish and Wildlife Research Unit[2], Department of Fisheries and Wildlife and U.S.G.S (for C.B.S), Oregon State University, Corvallis, OR 97331-3803, USA.

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Abstract

The present study investigated the effects of manipulating the GABAergic system on locomotor activity in juvenile spring chinook salmon, *Oncorhynchus tshawytscha*. In addition, we evaluated whether the GABAergic system is important for mediating the behavioral effects of corticotropin-releasing hormone (CRH). An intracerebroventricular (ICV) injection of the $GABA_A$ agonist muscimol caused an acute and dose dependent increase in locomotor activity in juvenile spring chinook salmon. ICV injections of the $GABA_A$ antagonist bicuculline prevented the increase in activity when administered concurrently with muscimol. The $GABA_B$ agonist baclofen had no effect on locomotor activity in this study. Furthermore, we found no evidence that the locomotor response to exogenous CRH was altered by the concurrent administration of muscimol or bicuculline. These results provide evidence to support the hypothesis that endogenous GABA within the central nervous system is involved in the control of locomotor activity in fish. The data also suggest that there is no interaction between the GABAergic system and CRH with regards to the control of locomotor activity in this species.

Introduction

Locomotion in teleosts, like other vertebrates, is a highly complex behavior. It involves the coordination of the motor system, which produces the propulsive movements, and the postural motor system, which maintains the body in the

appropriate orientation. The control of these systems in teleosts is relatively well understood, and is thought to be maintained by the brain stem and central pattern generators in the spinal cord (Grillner et al. 1998). However, the pathways and neurotransmitters involved in the expression of locomotor activity within the central nervous system (CNS) are poorly understood. To date most research on locomotor activity in teleosts has focused on the effects of serotonin (Genot et al. 1984; Winberg et al. 1993). However, dopamine (Kemnitz et al. 1995; Mok and Munro, 1998), corticotropin-releasing hormone (CRH) (Clements et al. In Press) and thyroid hormones (Hoar et al. 1955; Sage 1968) have also been implicated in the control of locomotor activity within the CNS. In mammals, GABAergic mechanisms are involved in the regulation of locomotor activity (Tirelli, 1989; Cazalets et al. 1994; Osborne, 1994). At least two receptor subtypes, $GABA_A$ and $GABA_B$, that differ in their pharmacological, electrophysiological and biochemical properties mediate the actions of GABA. Both subtypes are present in teleosts (Prunet et al. 1992; Berman and Maler, 1998) although little is known about their involvement in the control of locomotor behavior in the CNS

The behavioral effects of GABA in mammals appear to relate to both the dose and the site of injection (Tirelli, 1989). Plaznik et al. (1990) have reported that local injections of $GABA_A$ and $GABA_B$ agonists dose dependently decreased activity in rats. Similar results have been demonstrated following microinjection of muscimol into the posterior hypothalamus of domestic cats (Waldrop et al. 1988). In contrast, administration of the $GABA_A$ agonist muscimol into the medial septum or the

ventral hippocampus increased locomotor activity in laboratory rats (Alvarez and Banzan, 1990; Osborne, 1994).

Interactions between the GABAergic system and CRH have been evaluated *in vitro* (Jones, et al 1976, Calogero et al. 1988ab; Tizabi and Calogero, 1992). In general, GABA agonists inhibit the release of CRH or decrease the responsiveness of CRH neurons to stimulatory neurotransmitters. However, there is nothing known about the interaction between GABA and CRH in the control of locomotor activity. CRH is the initiating hormone in the hypothalamic-pituitary-interrenal response to stress, and is now thought to also play a role in integrating and coordinating the endocrine, neuroendocrine, autonomical and behavioral responses to stress (Vale, 1993). We have shown previously (Clements and Schreck, Submitted) that corticotropin releasing hormone (CRH) stimulates locomotor activity in juvenile chinook salmon, *Oncorhynchus tshawytscha*, when injected into the 3rd ventricle. Therefore, we hypothesized that GABA agonists would attenuate the increase in locomotor activity caused by CRH.

The objectives of the current study were: 1) to determine the effect of $GABA_A$ and $GABA_B$ agonists on locomotor activity in juvenile chinook salmon and 2) to determine if stimulation of the GABAergic system would attenuate the CRH induced increase in locomotor activity.

General Methods

Fish

Seven-month-old juvenile spring chinook (Willamette stock) (size: 92 ± 0.44 mm; mean ± 1 SEM) were held under natural photoperiod in a 336 L circular tank. Flow through water (12°C) was supplied from a well. Fish were fed twice daily with semi-moist pellet (BioOregon™). All experiments were conducted over a 3-week period beginning May 31, 1999 at Oregon State University's Fish Performance and Genetics Laboratory.

Chemicals

Muscimol ($GABA_A$ agonist), baclofen ($GABA_B$ agonist), bicuculline ($GABA_A$ antagonist), and ovine CRH were obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in teleost Ringers solution (0.2 % $NaHCO_3$ in 0.6 % NaCl solution). At the beginning of the study preparations of the appropriate concentrations were made by serial dilution of the stock in the Ringers solution. A fresh preparation was used on each day of testing. Both the stock solutions and the dilute preparations were stored frozen (-20°C) when not in use.

Administration procedure

Animals were netted from the holding tank and placed in anaesthetic until they lost equilibrium (50 mgL^{-1} tricaine methanesulfonate buffered with 125 mgL^{-1} $NaHCO_3$). Chemicals were administered freehand under a dissecting microscope

using a 10 μL glass syringe with a 26 G needle (Hamilton # 701). In preliminary trials this was found to be more accurate than using a stereotactic micrometer. Injections were performed midline, immediately behind the pineal gland, to a depth of 1-2 mm below the tissue surface. To prevent excess leakage of the chemicals the 2 μL volume was infused slowly and the needle was held in position for 5 s after the infusion to allow for clearance. The injection procedure took between 15-20 s per fish. The syringe and needle were rinsed with Ringers solution between animals and were cleaned with ethanol and Ringers following each days testing. The total injection volume was always 2 μL. Accuracy of injection into the 3rd ventricle was established in a preliminary trial and was approximately 85%.

Behavioral tests

Following the injection procedure fish were transferred to a testing arena and placed in a dark perforated plastic container for recovery. Fifteen min later the container was removed and the fish was able to swim freely in the arena. Activity was recorded for a 10 min period beginning at the time of release. The testing arenas consisted of a wooden tank, lined with dark green gelcoat. The inside dimensions were 150 cm x 31 cm x 21 cm (L x W x D). four identical arenas were used in these experiments. Well water (12°C) flowing at 7.5 L min^{-1} was introduced at the head of the tank and drained through a standpipe at the tail end. Disturbance was minimized by surrounding the tanks with black polyethylene curtains. Each arena was lit by a 100 watt incandescent bulb mounted 1.2 m above the water

surface. Swimming activity was monitored using 8 mm video cameras mounted 1.4 m above the water surface. During analysis the tank was divided into 8 equal segments by superimposing a grid onto the image. To compensate for parallax error the size of the segments was adjusted accordingly. Locomotor activity was quantified by counting the number of line crossings per unit time. A line crossing was recorded when the fish crossed any line on the grid while actively swimming/gliding in a forward direction. Stereotyped circling or spiraling behavior was not recorded as activity.

Statistical Analysis

Data from all treatments was normally distributed ($P > 0.05$, Kolmogorov-Smirnov test). However, all data were analyzed using non-parametric methods because of unequal variances between treatments and small sample sizes. Group differences were analyzed using a Kruskal-Wallis test. Treatment differences were analyzed using Dunn's multiple range post-test. In experiment 3 only planned comparisons were analyzed. Differences between days within a treatment were analyzed using the Mann-Whitney *U* test.

Specific Methods and Results

Experiment 1: Dose response study: ICV injections of muscimol

To determine if the $GABA_A$ receptor subtype was involved in the regulation of locomotor behavior we administered either saline or muscimol at 3 doses (1, 10 or 50 ng). Thirty animals were injected in each treatment group. The experiment was conducted over 2 days. There was no difference in median activity levels within any of the treatment groups over the two days; therefore, we combined data within each treatment. ICV injections of muscimol stimulated a dose dependant increase in locomotor activity (Fig. IV.1). The activity of fish injected with muscimol was significantly higher than in the control group at the 10 ng ($P<0.01$) and 50 ng ($P< 0.001$) doses.

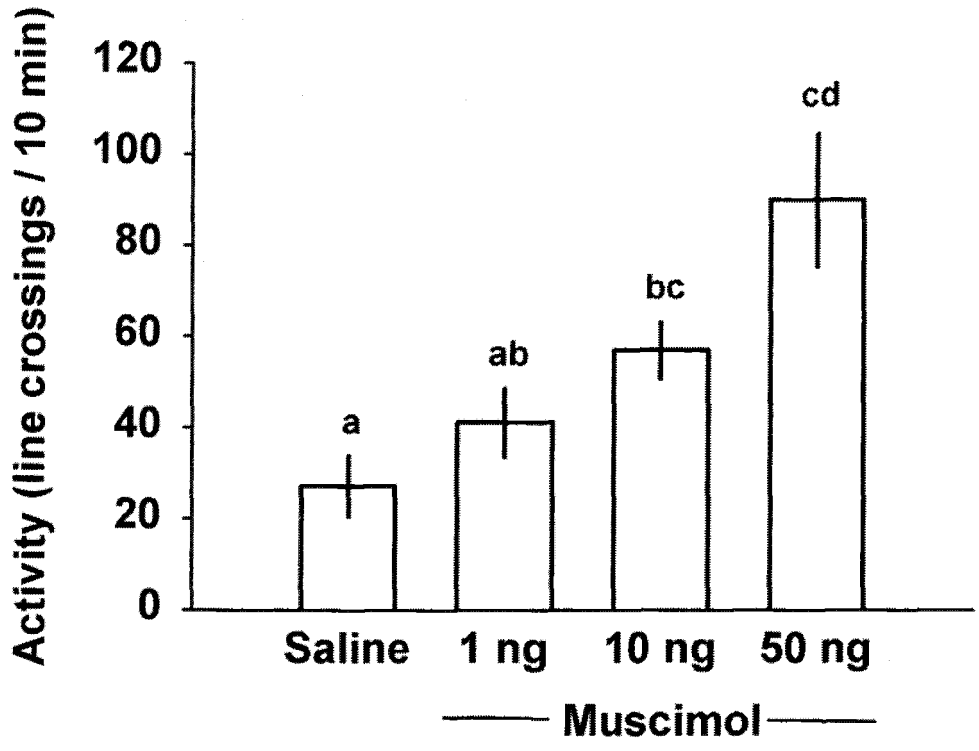

Figure IV.1 Locomotor activity in juvenile chinook salmon following ICV injections of saline or muscimol (1, 10, 50 ng). Locomotor activity was measured by placing fish in a rectangular tank that was divided into 8 equal size quadrants. Each column represents the mean (± 1 S.E.M.) number of line crossings over a 10 min period starting 15 min post-injection. Columns that share a common superscript are not different ($p > 0.05$, Dunn's multiple range test). $N = 30$ for all treatments

Experiment 2: Dose response study: ICV injections of baclofen

To determine if the $GABA_B$ receptor subtype was involved in the regulation of locomotor behavior we administered either saline or baclofen at 3 doses (1, 10 or 100 ng). Twenty animals were injected in each treatment group. The experiment was conducted over 2 days. There was no difference in activity levels within any of the treatment groups over the two days therefore we combined data within each

treatment. ICV injections of baclofen at the 2 lower doses had no effect on locomotor activity in this environment (Fig. IV.2). However, at the highest dose (100 ng), there was a high incidence of abnormal swimming behavior. Therefore, testing of fish at this dose was terminated after 7 fish.

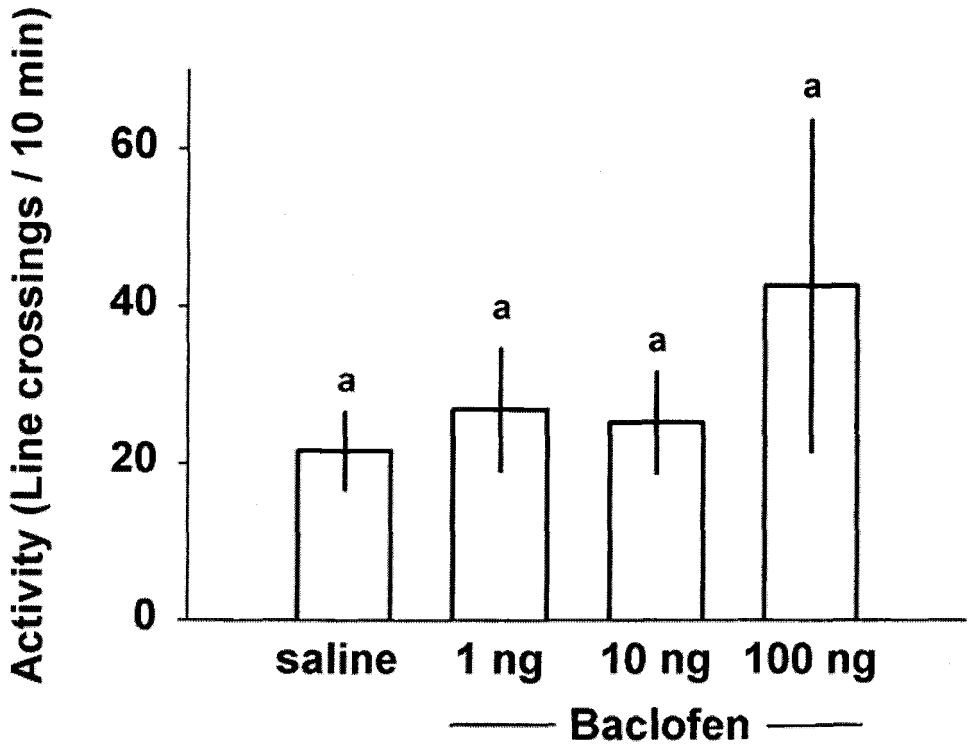

Figure IV.2 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline or baclofen (1, 10, 100 ng). Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($p > 0.05$, Dunn's multiple range test). $N = 20$ for all treatments except the treatment group receiving 100 ng baclofen where $N = 7$.

Experiment 3: Effect of the $GABA_A$ antagonist bicuculline

To determine if blocking the binding of endogenous GABA to the $GABA_A$ receptor would decrease locomotor activity we administered bicuculline at 2 doses (1 or 10 ng). We also tested whether the stimulatory effects of muscimol were due to the specific activation of the $GABA_A$ receptor by administering muscimol (10 ng) alone or concurrently with bicuculline (10 ng). All treatments were compared to a saline injected control group. Twenty fish were injected in each treatment group. During the data analysis the experiment was divided into two parts: the first compared the two bicuculline injected treatment groups and the control group; the second compared the control, muscimol, and the muscimol/bicuculline treatments.

Bicuculline did not decrease locomotor activity compared to saline injected controls (Fig. 3). However, bicuculline markedly attenuated the muscimol-induced increase in locomotor activity when administered together ($P<0.01$) (Fig. IV.3).

Experiment 4: Interaction between muscimol and CRH

To determine if the stimulatory effects of CRH previously demonstrated are modulated by the GABAergic system we injected animals with either saline, muscimol (10 ng), CRH (500 ng), or muscimol 10 ng/CRH 500 ng concurrently. Twenty fish were injected in each treatment group.

Both muscimol and CRH stimulated an increase in locomotor activity ($P< 0.001$) (Fig. IV.4). However, there was no further change in activity when muscimol was administered concurrently with CRH.

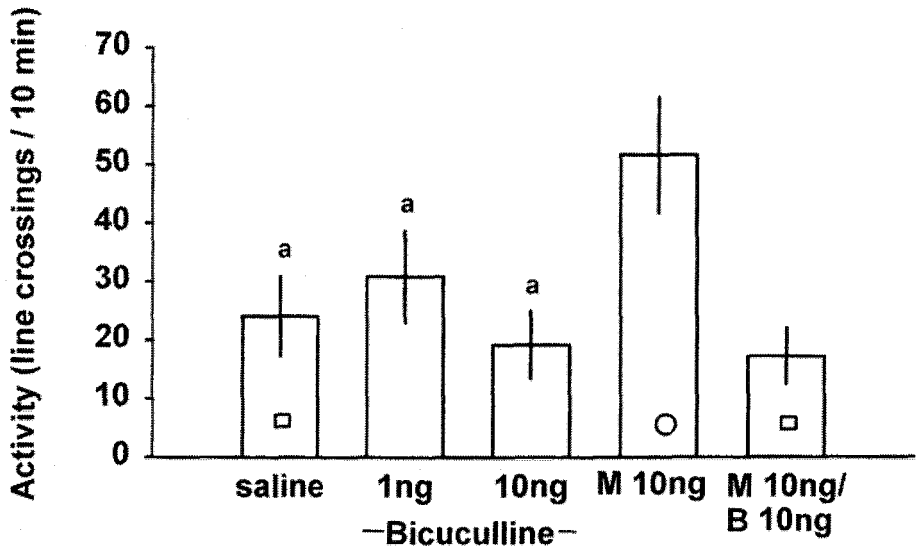

Figure IV.3 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline, bicuculline (1 or 10 ng), muscimol (M 10 ng) or a combination of bicuculline and muscimol (M 10 ng / B 10 ng). Locomotor activity was measured as described in figure 1. Saline, 1ng and 10 ng treatments were compared. Columns that share a common superscript are not different ($p > 0.05$, Dunn's multiple range test). Saline, M 10 ng and M 10 ng/B 10 ng treatments were compared. Columns that share a common symbol are not different ($p > 0.05$, Dunn's multiple range test). $N = 20$ for all treatments.

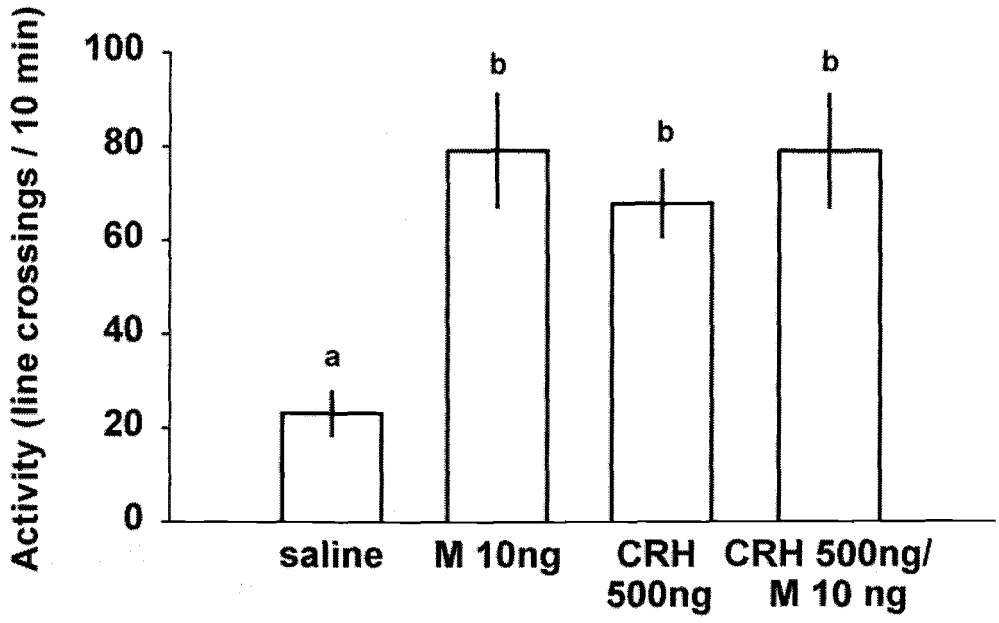

Figure IV.4 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline, muscimol (M 10 ng), CRH (500 ng) or a combination of CRH and muscimol (CRH 500 ng / M 10 ng). Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($p > 0.05$, Dunn's multiple range test). $N = 20$ for all treatments.

Experiment 5: *Interaction between bicuculline and CRH*

To further investigate possible interactions between CRH and GABA in the control of locomotion endogenous GABAergic activity was attenuated. Four treatment groups were tested; saline, bicuculline 10 ng, CRH 500 ng, or bicuculline 10 ng and CRH 500 ng concurrently. Twenty fish were injected for each treatment group. CRH stimulated locomotor activity ($P < 0.01$), but the effect was not enhanced by concurrent treatment with bicuculline (Fig. IV.5).

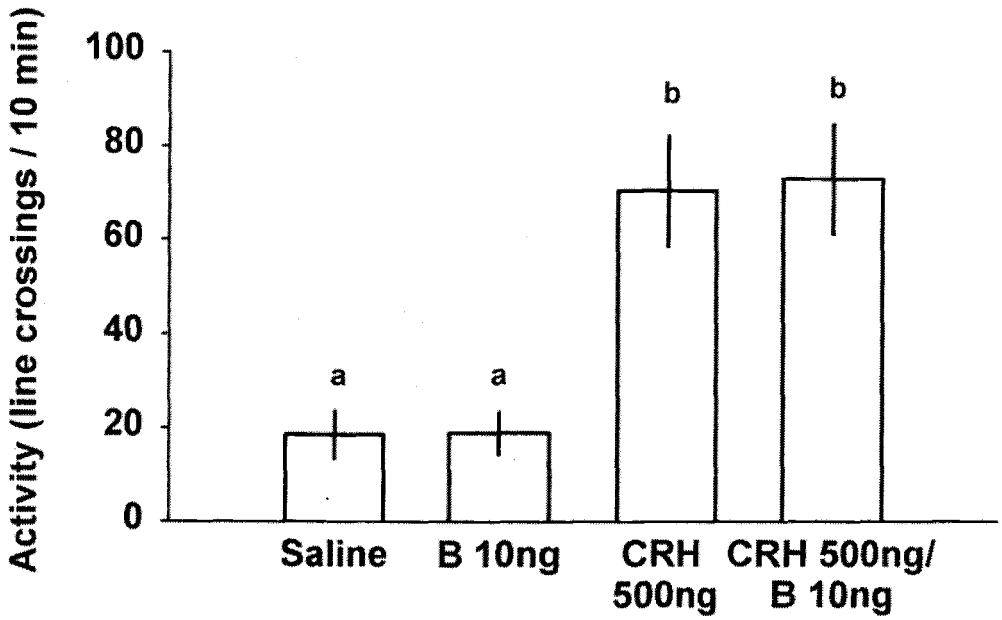

Figure IV.5 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline, bicuculline (B 10 ng), CRH (500 ng) or a combination of CRH and bicuculline (CRH 500 ng / B 10 ng). Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($p > 0.05$, Dunn's multiple range test). $N = 20$ for all treatments.

Discussion

These results suggest that activation of endogenous GABAergic mechanisms stimulates locomotor activity in juvenile chinook salmon. ICV administration of muscimol induced a significant, dose dependent increase in locomotor activity in juvenile chinook. This effect is most likely mediated by the binding of muscimol to the $GABA_A$ receptor complex as ICV injections of baclofen, a $GABA_B$ receptor agonist, failed to induce any increase in activity. Furthermore, the concurrent administration of the $GABA_A$ receptor antagonist bicuculline with muscimol

significantly attenuated the increased locomotor activity compared to muscimol alone.

The third ventricle was chosen as the site of injection in this study because of its proximity to brain centers that are involved in the control of locomotion, and the increased likelihood of diffusion to a greater area of the brain than other injection sites. In fishes, the third ventricle is located in the diencephalon but continues into the mesencephalon via the aqueduct of Sylvius (Takashima and Hibiya, 1995). Injections into the third ventricle are therefore most likely to affect the areas of the diencephalon, including the preoptic nucleus, nucleus preopticus magnocelluraris, nucleus preopticus parvocellularis. It is also possible that diffusion into the thalamus, and other regions of the hypothalamus occurs. However the most likely site for causing the behavioral effects observed in this study is the mesencephalon. This region contains the integration centers for the visual and other senses and locomotion. GABA immunoreactive cells have been identified in the mesencephalon in the 3-spined stickleback, *Gasterosteus aculeatus*, (Ekstrom and Ohlin, 1995) while the $GABA_A$ receptor has also been localized in the mesencephalon of Atlantic salmon, *Salmo salar*, (Anzelius et al. 1995). CRH expressing cells have been identified in the mesencephalon of gilthead sea bream, *Sparus aurata*, (Mancera and Fernandez-Llebrez, 1995). However, these cells appear to occur at higher densities in the preoptic nucleus, for example, in rainbow trout (Hasegawa et al. 1999), carp and goldfish (Olivereau et al. 1984). Given the current state of our knowledge about the distribution of CRH expressing cells and

cells and terminal fibers in the brain of fish, and their relation to monoaminergic cells there are many possible scenarios for the behavioral effects of CRH. These might include the release of CRH into the ventricle from the nucleus preopticus and transport to the motor control regions or termination of CRH fibers onto monaminergic cells within the motor control regions.

The increased locomotor activity we observed was characterized by a general increase in sustained forward swimming behavior rather than increased incidents of burst swimming. However, at a dose of 100 ng injections of both muscimol (results not shown) and bicuculline caused the fish to swim in a spiral pattern. This behavioral pattern lasted for 40-50 min before the fish resumed normal swimming. Takeuchi (1994) reported that unilateral infusion of muscimol or baclofen into the posterior thalamus induced contralateral (turning to the side opposite the infusion) circular swimming in the medaka *Oryzias latipes*. Furthermore, the administration of $GABA_A$ and $GABA_B$ antagonists induced ipsilateral circular swimming (turning towards the infused side). In the current study several observations were made of fish exhibiting this stereotyped circling behavior following injections of muscimol or bicuculline. The behavior involved the fish swimming in tight circles to the left or right for up to 20 min continuously. It is not clear, however, whether the circling or spiraling behavior observed in this study was due to the effects of GABA on postural or steering control or simply due to seizures caused by the highest dose. In the current study locomotor activity was quantified over a 10 min period beginning 15 min after release. Recording of behavior was not done during the first 15 min to

allow for recovery of normal swimming behavior following anaesthesia, and to allow time for the diffusion of the chemicals. However, in preliminary trials the locomotor stimulating effects of both muscimol and CRH were observed immediately upon recovery from anaesthesia and extended to over 1 h for muscimol and at least 24 h for CRH (Clements et al. In Press). The timecourse for the effects of both these chemicals is also likely to depend on the dose.

The role GABAergic pathways within the CNS in controlling specific locomotor behaviors is unclear. At the cellular level GABA is an inhibitory neurotransmitter that acts to prevent firing in the post-synaptic cell. Indeed, both $GABA_A$ and $GABA_B$ agonists diminish rhythmic locomotor-like activity in spinal preparations of laboratory rats (Cazalets et al. 1994) and adult silver lamprey, *Ichthyomyzon unicuspis*, (Tegner et al. 1993). However, there is evidence that GABA can elicit both inhibitory and excitatory (by disinhibition) actions within the central neurons (Yaravsky and Carpenter, 1978; Walker, 1986; Evangelista et al. 1987). At the organismic level the administration of muscimol both systemically and into the CNS caused increased locomotor activity in rats (Alvarez and Banzan, 1990; Osborne et al. 1993; Osborne, 1994) and mice (Tirelli, 1989). It is likely that the stimulatory action of GABA occurs due to disinhibition, possibly of the dopaminergic system. (Evangelista et al. 1987; Clements and Schreck, submitted). Furthermore, the effects may relate to both the dose and site of administration. Low doses of muscimol given systemically appear to cause activation of locomotor

activity while high doses result in the inhibition of locomotor activity (Tirelli, 1989).

The increase in activity following ICV administration of CRH we report in this study is in agreement with previous studies on fish (Clements and Schreck, Submitted), amphibians (Moore et al. 1984), and mammals (Sutton, 1982). The stimulating effect of CRH on locomotor activity appears to be mediated via extrahypothalamic regions of the brain, where to our knowledge the interaction between GABA and CRH has not been studied. However, several authors have reported the occurrence of interactions between GABA and CRH within the hypothalamus. In general, the release of CRH within the hypothalamus is inhibited by innervation from GABAergic fibers. GABA may also be co-stored with CRH and released simultaneously, possibly to prevent excess release of CRH (Grossman et al. 1993).

We hypothesized that endogenous GABA might oppose stress related behaviors, including locomotor activity, by inhibiting CRH within the motor regions of the CNS. However, ICV administration of muscimol or bicuculline had no effect on locomotor activity induced by CRH. The absence of an effect between CRH and bicuculline suggests that endogenous GABA does not inhibit the action of CRH as we would have expected to see an increase in activity compared to fish given CRH alone. Although the current results do not suggest any interaction, it is possible that the dose used (10 ng) was too low, that endogenous GABA inhibits synthesis rather than release of CRH or that the system was already overstimulated

by the administration of exogenous CRH. However, if the latter were the case, we would have expected the administration of bicuculline alone to increase activity in these fish due to the inhibition of endogenous CRH release. We have demonstrated that the administration of a CRH antagonist decreases activity levels in this species. This suggests that the level of locomotor activity in controls is, in part, a function of endogenous CRH release (Clements et al. Submitted). In the future, *in vitro* preparations may be useful for isolating a potential interaction between GABA and CRH within the motor regions of the CNS as it is possible to achieve a greater level of control within the system.

Changes in locomotor activity can occur for a variety of reasons, including foraging, predator avoidance and migrations. The absence of an additive effect between muscimol and CRH suggests that their stimulatory effect on locomotor activity occurs independently. Therefore endogenous GABA and CRH might mediate locomotor activity during unique situations. In general, CRH tends to enhance behavioral responses to stress (Koob et al. 1993), and increase arousal, the effect being dependant on the dose and the environmental context. In contrast, endogenous GABA is almost certainly the major inhibitory neurotransmitter within the CNS. The current experimental design was such that we cannot distinguish between different goal oriented increases in activity. Therefore, further investigation is required using alternative behavioral tests to determine the purpose of the increased activity associated with CRH and GABA.

In summary this study provides evidence that endogenous GABA is involved in the control of locomotor activity in juvenile chinook salmon. Furthermore, the increase in locomotor activity observed is mediated by the $GABA_A$ receptor complex. Despite *in vitro* studies suggesting that GABA inhibits the release of CRH within the hypothalamus we found no behavioral evidence for any interaction between GABA and CRH.

Acknowledegments

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CHAPTER V

EVIDENCE THAT GABA MEDIATES DOPAMINERGIC AND SEROTONINERGIC PATHWAYS ASSOCIATED WITH LOCOMOTOR ACTIVITY IN JUVENILE CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)[1].

Shaun Clements and Carl B. Schreck

Oregon Cooperative Fish and Wildlife Research Unit[2], Department of Fisheries and Wildlife and U.S.G.S (for C.B.S), Oregon State University, Corvallis, OR 97331-3803, USA

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Abstract

The control of locomotor activity in juvenile chinook salmon, *Oncorhynchus tshawytscha*, was examined by manipulations of 3 neurotransmitter systems; δ-amino-n-butyric acid (GABA), dopamine (DA), serotonin (5-HT) and the neuropeptide corticotropin-releasing hormone (CRH). In agreement with previous studies, intracerebroventricular (ICV) injections of the $GABA_A$ agonist muscimol and CRH stimulated locomotor activity. The stimulating effect of muscimol was attenuated by concurrent administration of a DA receptor antagonist, haloperidol. Furthermore, the concurrent administration of a dopamine uptake inhibitor (4',4"-Difluoro-3alpha-(diphenylmethoxy) tropane Hydrochloride) potentiated the effect of muscimol on locomotor activity. In contrast haloperidol had no effect on locomotor activity when administered concurrently with CRH. These results support the hypothesis that the stimulatory effect of muscimol is produced by the activation of dopaminergic systems. They further suggest that the effect of CRH on locomotor activity is not directly mediated by the dopaminergic system.

ICV injections of muscimol either had no effect or attenuated the locomotor response to concurrent injections of CRH and fluoxetine (5-HT selective reuptake inhibitor). Conversely, an ICV injection of the $GABA_A$ antagonist, bicuculline methiodide, significantly potentiated the increase in locomotor activity observed following the concurrent administration of CRH and fluoxetine. Previous studies suggest that GABA does not directly inhibit the locomotor response to CRH. Based

on these results we speculate that endogenous GABA may indirectly regulate behaviors associated with CRH release by inhibiting the serotonergic system.

Introduction

Fish often exhibit complex and variable behavioral and physiological responses to similar stressors. A mechanism that is sensitive to input from multiple systems would allow this variability in both the intensity and type of behavioral and physiological output. A number of studies on vertebrates have shown that multiple neurotransmitter systems within the brain regulate both behavioral and physiological output during stress, or indeed at any time (Bradford, 1986). Such behaviors include, reproduction, feeding, and locomotor activity. Among fishes however, there is limited understanding of the neural mechanisms for controlling behavior.

Previously we have shown that the $GABA_A$ agonist muscimol and the neuropeptide CRH stimulate locomotor activity in juvenile chinook salmon (*Oncorhynchus tshawytscha*) (Clements and Schreck, 2001; Clements et al. In Press). Given that GABA functions primarily as an inhibitory neurotransmitter, we hypothesized that the stimulatory effect on locomotor activity was due to the disinhibition of another neurotransmitter system. There is some evidence that increases in locomotor activity in mammals following administration of GABA agonists can be attenuated by administration of dopamine (DA) antagonists (Osborne et al. 1993). Dopamine agonists also have stimulatory effects on

locomotor activity. Mok and Monro (1998) reported that the addition of apomorphine to the water significantly increased locomotor activity in tilapia (*Oreochromis niloticus* and *O. mossambicus*). Furthermore, this effect was abolished by the addition of DA antagonists to the water.

We had previously hypothesized that endogenous GABA would also directly inhibit the stimulatory effect of CRH on locomotor activity (Clements and Schreck, 2001a). In that study, however, GABA agonists and antagonists had no effect on locomotor activity when given concurrently with CRH. Conversely, the stimulatory effect of CRH appears to be facilitated by the serotonergic system (Clements et al. Submitted). Previous studies have shown that GABA inhibits serotonergic activity within the central nervous system of mammals (Nishikawa and Scatton, 1985a,b; Wirtshafter et al. 1988). Similarly, it appears that dopamine is often antagonistic to the effects of serotonin within the nucleus accumbens and the nigrostriatal system. Nigrostriatial dopamine pathways are directly involved in locomotion (Bradford 1986). We therefore hypothesized that GABA and/or DA would indirectly inhibit the locomotor response to CRH by modifying the output of the serotonergic system.

The objectives of this study were (1) to determine whether the stimulatory effect of GABA on locomotor activity is linked to the activation of dopaminergic systems, (2) to determine whether dopaminergic systems are involved in the regulation of locomotor activity that is under the control of CRH, and (3) to

determine whether endogenous GABA indirectly controls CRH mediated activity by inhibition of the serotonergic system.

General Methods

Fish

The fish in experiments 1-7 were 9-11 month old juvenile spring chinook salmon parr (114.3 ± 1.6 mm in length) (Marion Forks stock). The fish in part 1 of experiment 6 were 14 month old juvenile spring chinook salmon parr (131.1 ± 0.9 mm in length) (Willamette River stock). Fish were held under ambient photoperiod in 336 L circular tanks at Oregon State University's Fish Performance and Genetics Laboratory. Flow through water (12°C) was supplied from a well. Fish were fed twice daily with semi moist pellet (BioOregon™).

Chemicals

Muscimol ($GABA_A$ agonist), bicuculline methiodide ($GABA_A$ antagonist), ovine CRH, haloperidol ($DA_{2,3,4}$ selective antagonist), 4',4"-Difluoro-3alpha-(diphenylmethoxy) tropane Hydrochloride (DUI; DA selective uptake inhibitor) and fluoxetine (5-HT selective re-uptake inhibitor) were obtained from Sigma Chemical Co. (St. Louis, MO). Due to the insoluble nature of DUI it was first dissolved in DMSO then diluted 1:10 in 1.1 X teleost Ringers solution (0.22 % $NaHCO_3$ in 0.66 % NaCl solution) to maintain the appropriate molality of the final solution. The remaining chemicals were dissolved in standard teleost Ringers

solution. (0.20 % $NaHCO_3$ in 0.6 % NaCl solution). At the beginning of each experiment aliquots of the appropriate concentrations were made by serial dilution of the stock in Ringers solution. A fresh aliquot was used on each day of testing. Both the stock solutions and aliquots were stored frozen (-20°C) when not in use.

To test the effects of the DMSO vehicle on control behavior in experiment 4 a modified Ringers solution was made by diluting DMSO 1:10 in 1.1 X Ringers solution. In addition, muscimol (50 ng) was dissolved in this modified Ringers to test the effect of DMSO on induced hyperactivity.

Administration procedure

Fish were netted from the holding tank and anaesthetized (5-7 min in 50 mgL^{-1} tricaine methanesulfonate buffered with 125 mg L^{-1} $NaHCO_3$). Chemicals were administered following a procedure described previously (Clements and Schreck, 2001). In summary, injections were performed midline, immediately behind the pineal gland into the 3rd ventricle. The needle was inserted to a depth sufficient to penetrate the cartilaginous brain casing and enter the 3rd ventricle. The total injection volume was 2 μL. A preliminary trial established the accuracy of this procedure as approximately 85%.

Behavioral testing

Fish were injected and tested individually. The assignment of treatments and the arena for testing were random. Following the injection each fish was transferred

to the testing arena and placed in a dark perforated plastic container for recovery. Fifteen min later the container was removed and the fish was able to swim freely in the arena.

For experiment 1, the arena consisted of a wooden tank, lined with dark green gelcoat. The inside dimensions were 150 cm x 31 cm x 21 cm (L x W x D). Four identical arenas were used in these experiments. Well water (12°C) flowing at 7.5 L min^{-1} was introduced at the head of the tank and drained through a standpipe at the tail end. Visual disturbance was minimized by surrounding the tanks with black polyethylene curtains. Each arena was lit by a 100 watt incandescent bulb mounted 1.2 m above the water surface. For this experiment, activity was monitored from above by 8 mm video cameras for a 10 min period beginning at the time of release. During analysis the tank was divided into 8 equal segments by superimposing a grid onto the recorded image.

For experiments 2-7 the testing arena consisted of a light blue fiberglass tank with inside dimensions of 965 mm x 965 mm x 609 mm. Two identical arenas were used. Each arena was filled with well water to a depth of 10 cm. The water was replaced by draining and refilling the tank following observations on 3-4 fish to maintain high water quality and ensure the temperature was within 0.5 ^{0}C of the holding tanks. Each arena was lit by two 100 watt incandescent bulbs mounted 2.28 m above the water surface. For these experiments, activity was monitored from above by 8 mm video cameras for a 10 min period beginning at the time of release.

During analysis the tank was divided into 36 equal segments by superimposing a grid onto the recorded image.

For all experiments, activity was quantified by counting the number of line crossings during this period. Line crossings were recorded when the fish was actively swimming/gliding in a forward direction. Stereotyped circling behavior was sometimes observed, most likely due to injection directly into the optic lobes. These fish were eliminated from the analysis.

Statistical Analysis

All data were analyzed using non-parametric methods because of unequal variances between treatments. Treatment differences were analyzed using a Kruskal-Wallis test. Treatment differences were analyzed using Dunn's multiple range post-test. Only planned comparisons were considered in the post tests. Differences between days within a treatment; and differences between the saline and muscimol treatments and their respective DMSO controls in experiment 4 were analyzed using the Mann-Whitney *U* test.

Specific Methods and Results

Experiment 1: Dose response study: the effect of acute treatment with DUI

To determine if enhancing endogenous dopaminergic activity increases locomotor activity, fish were injected ICV with either saline or DUI (10, 100, 1000 ng). Fifteen fish were injected in each treatment group. The experiment was

conducted on a single day in November 2000. ICV injections of DUI significantly increased locomotor activity at the highest dose ($P < 0.001$) (Fig. V.1).

Experiment2: Interaction between muscimol and DUI

To determine if the stimulatory effect of muscimol on locomotor activity was facilitated by dopaminergic activity, fish were injected ICV with either saline, muscimol (10 ng), or muscimol (10 ng) and DUI (10 or 100 ng) concurrently. Fifteen fish were injected in each treatment group. The experiment was conducted over 2 days in November 2000. There was no difference in activity levels within any of the treatment groups over the two days; therefore, we combined data within each treatment. In agreement with previous results an ICV injection of muscimol stimulated locomotor activity ($P < 0.05$) (Fig V.2). The concurrent administration of DUI (100 ng) with muscimol significantly potentiated the increase compared to muscimol alone ($P < 0.01$).

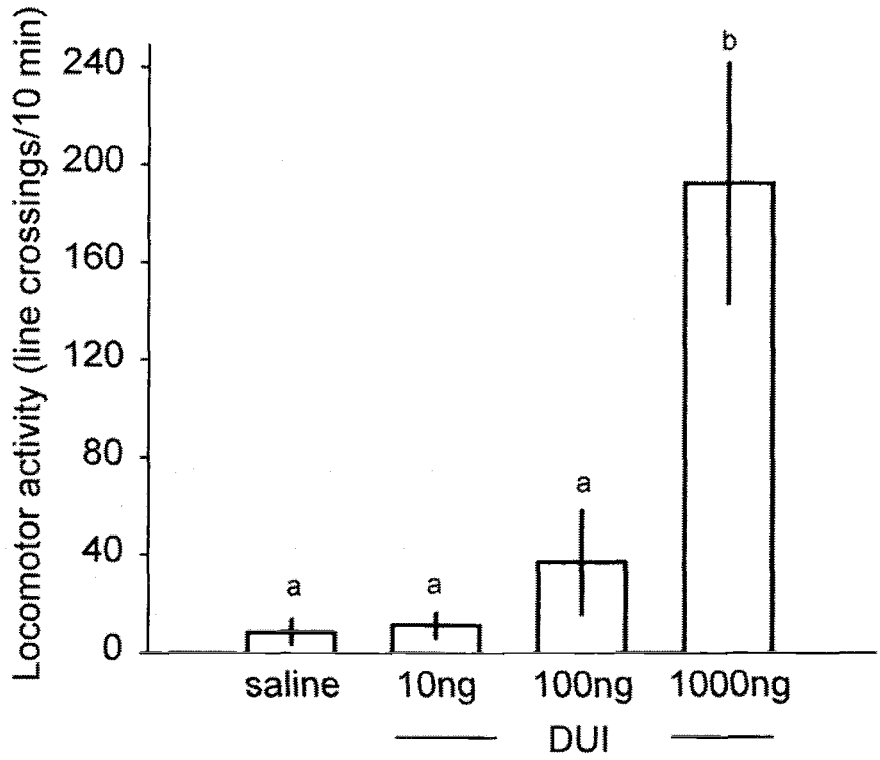

Figure V.1 Locomotor activity in juvenile chinook salmon following ICV injections of saline or DUI (10, 100, 1000 ng). Locomotor activity was measured by placing fish in a square tank that was divided into 36 equal size quadrants. Each column represents the mean (± 1 S.E.M.) number of line crossings over a 10 min period starting 15 min post-injection. Columns that share a common superscript are not different ($P > 0.05$, Dunn's multiple range test). $N = 15$ for all treatments.

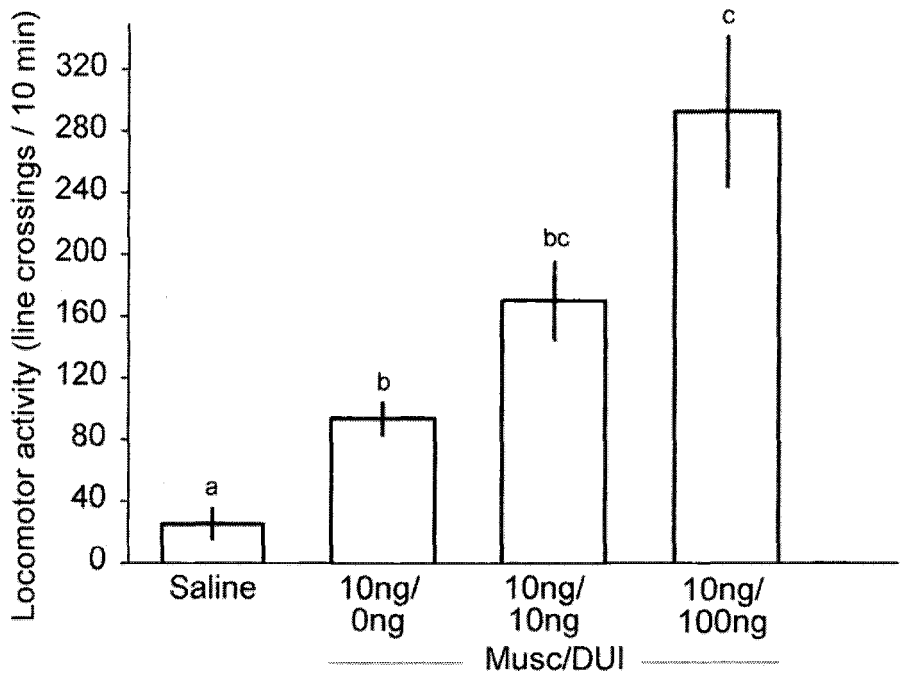

Figure V.2 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline, muscimol (10 ng) or a combination of muscimol (10 ng) and DUI (10 or 100 ng). Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($P > 0.05$, Dunn's multiple range test). $N = 15$ for all treatments.

Experiment 3: Dose response study: the effect of acute treatment with haloperidol

To further investigate the role of dopaminergic activity on locomotor activity, fish were injected ICV with either saline or haloperidol (10, 100, 500 ng). Fifteen fish were injected in each treatment group. The experiment was conducted on a single day in September 1999. ICV injections of haloperidol had no effect on locomotor activity at any of the 3 doses (Fig. V.3).

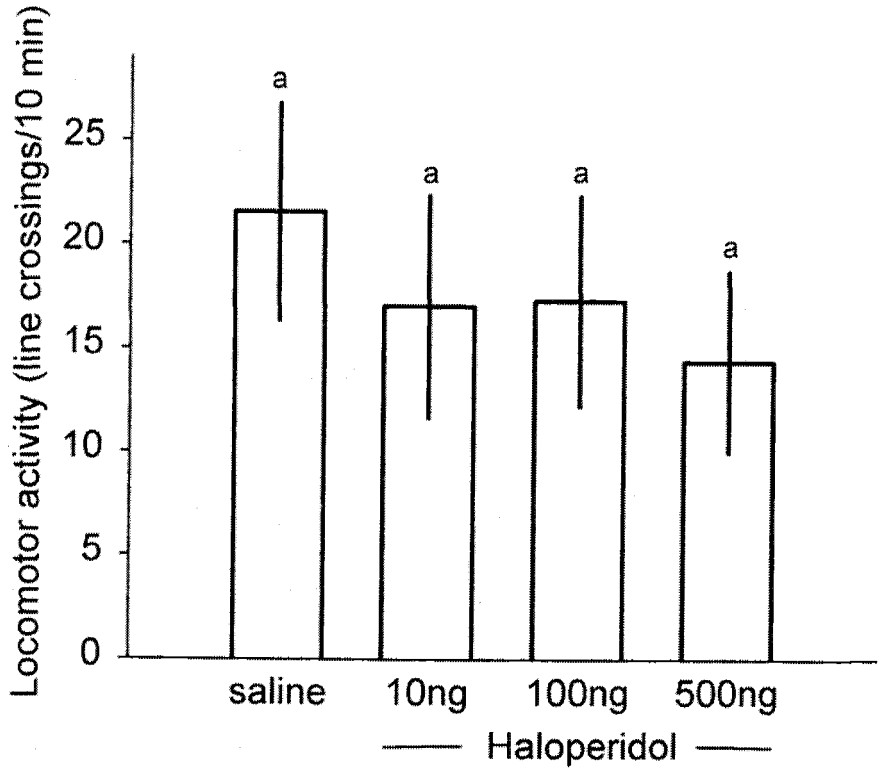

Figure V.3 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline or haloperidol (10, 100, 500 ng). Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($P > 0.05$, Dunn's multiple range test). $N = 15$ for all treatments.

Experiment 4: Interaction between muscimol and haloperidol

To further determine if the effect of muscimol on locomotor activity is due to the activation of dopaminergic mechanisms, fish were injected ICV with either muscimol (50 ng) or a combination of muscimol (50 ng) and haloperidol (10, 50 or 100 ng). All treatments were compared to a saline injected control group. The effect of the DMSO vehicle on locomotor behavior was evaluated. Saline controls

were compared to fish injected ICV with the modified Ringers solution described previously. In addition, the effect of DMSO on induced hyperactivity was determined by comparing the treatment group given muscimol dissolved in standard Ringers solution to a second group given muscimol dissolved in the modified Ringers solution containing DMSO. Twenty fish were injected in each treatment group. The experiment was conducted over 2 days in September 1999. There was no difference in mean activity levels within any of the treatment groups over the two days, therefore we combined data in each treatment. DMSO had no effect on control or muscimol-induced levels of activity. Therefore, all further comparisons were made to the original saline and muscimol treatment groups that did not receive DMSO.

ICV injections of muscimol stimulated locomotor activity ($P < 0.001$) (Fig V.4). The concurrent administration of haloperidol (50 and 100 ng) with muscimol tended to attenuate the effect of muscimol alone but the effect was only significant at 100 ng ($P < 0.001$).

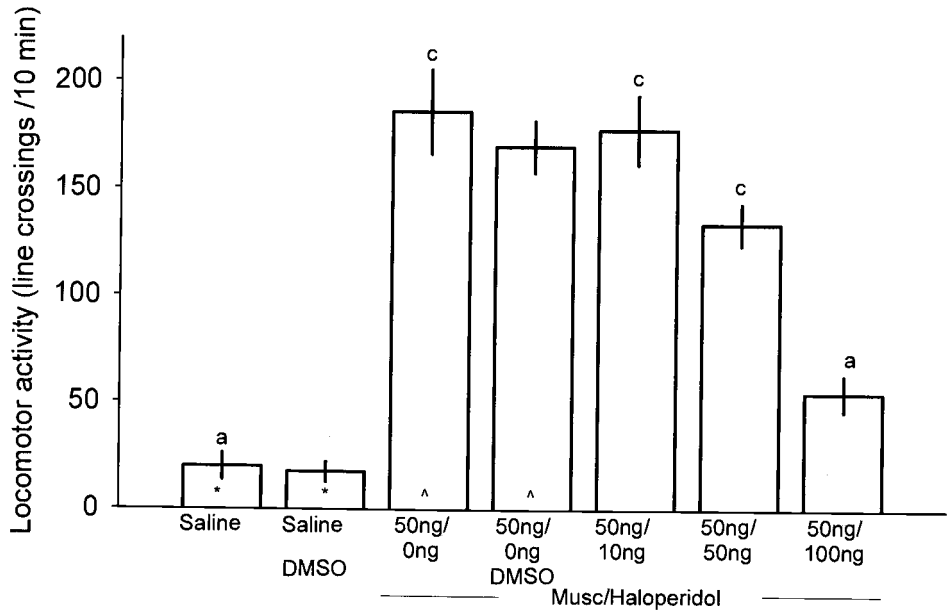

Figure V.4 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline, a modified ringers solution containing DMSO (DMSO), muscimol (50 ng), muscimol dissolved in the modified ringers solution (Musc 50 ng/ DMSO), or a combination of muscimol (50 ng) and haloperidol (10, 50 or 100 ng). Locomotor activity was measured as described in figure 1. Saline and muscimol (500ng) were compared to their respective treatments with DMSO. Columns that share a common symbol are not different ($p > 0.05$, Mann Whitney U test). Saline, muscimol (50 ng) and haloperidol (10, 50, 100 ng) were compared. Columns that share a common superscript are not different ($p > 0.05$, Dunn's multiple range test). $N = 20$ for all treatments.

Experiment 5: Interaction between CRH and haloperidol

To determine if the stimulation of locomotor activity due to the administration of CRH is affected by the dopaminergic system, fish were injected ICV with either saline, CRH (500ng) or a combination of CRH (500ng) and haloperidol (10 or 100 ng). Twenty fish were injected in each treatment group. The experiment was conducted over 2 days in September 1999. There was no difference in activity

levels within any of the treatment groups over the two days; therefore, we combined data within each treatment. In agreement with the previous results an ICV injection of CRH stimulated locomotor activity ($P < 0.001$) (Fig 5). The concurrent administration of haloperidol with CRH had no effect on locomotor activity at either dose compared to CRH alone.

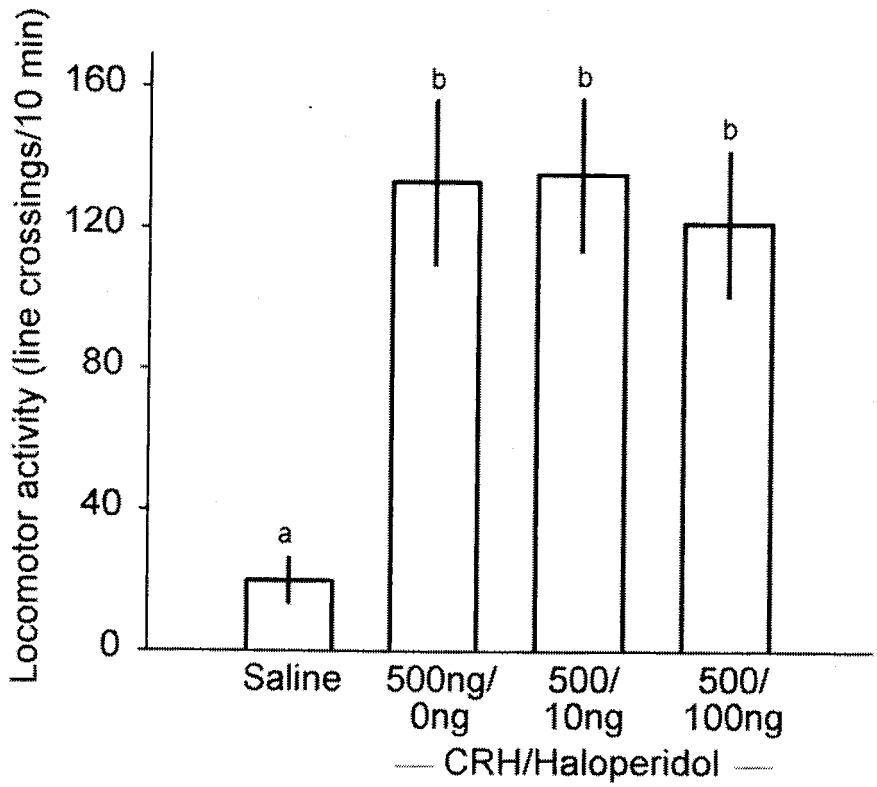

Figure V.5 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline, CRH (500 ng), or a combination of CRH (500 ng) and haloperidol (10 or 100 ng). Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($P > 0.05$, Dunn's multiple range test). $N = 20$ for all treatments.

Experiment 6: Effect of muscimol on fluoxetine/CRH induced hyperactivity

The effect of GABAergic enhancement on the stimulatory effect of CRH and serotonin was evaluated. Fish were injected ICV with either saline, CRH (500ng), a combination of CRH (500 ng) and fluoxetine (100ng) or CRH (500 ng), fluoxetine (100 ng) and muscimol (10 or 50 ng). This experiment was repeated 3 times. The results varied significantly, therefore the results of each experiment are presented separately. The first experiment was conducted on a single day in Jan 1999 ($N = 20$ for all treatments). An ICV injection of CRH and fluoxetine stimulated locomotor activity ($P < 0.001$)(Fig V.3). The concurrent administration of muscimol with CRH and fluoxetine significantly ($P < 0.05$) attenuated the effect (Fig V.6a). The experiment was repeated in October 2000. On this occasion muscimol had no effect on the activity following a combined injection of CRH and fluoxetine (Fig V.6b) ($N = 10$ for all treatments. The experiment was again repeated in November 2000 with similar results to the October trial (Fig V.6c) ($N = 20$ for all treatments).

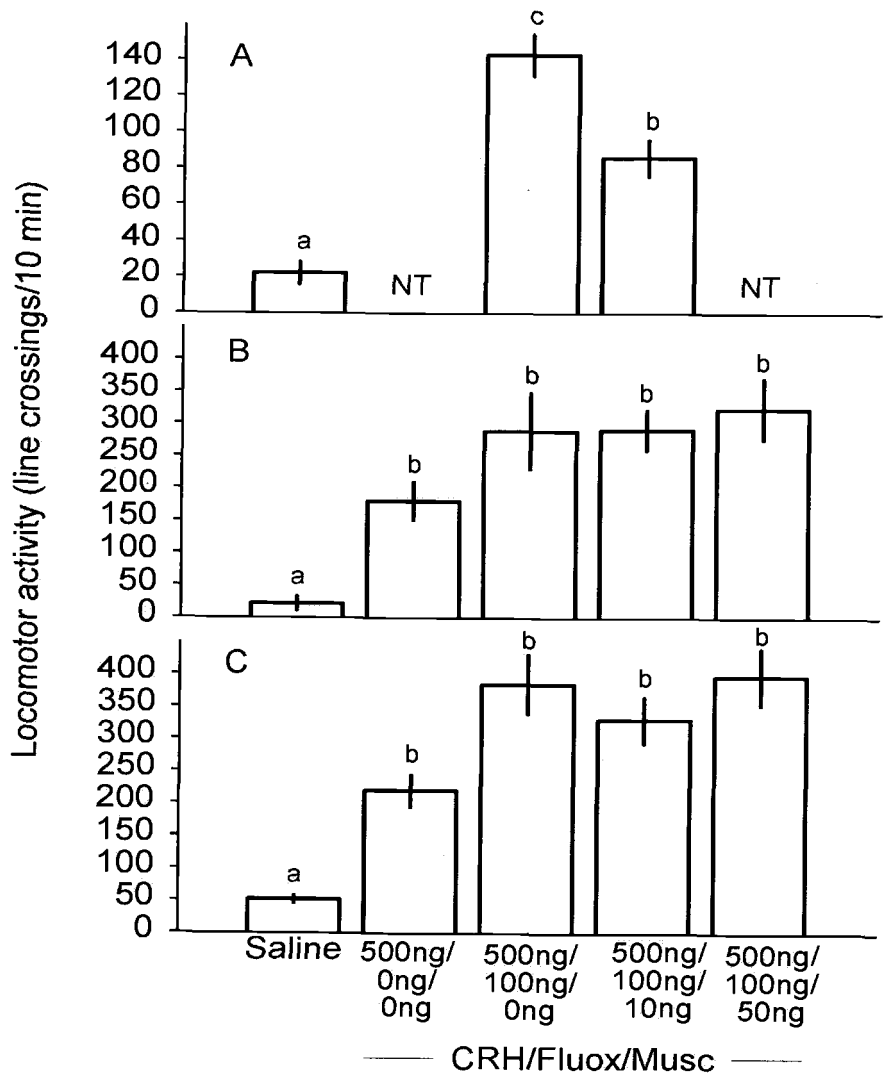

Figure V.6 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline, CRH (500 ng), a combination of CRH (500 ng) and fluoxetine (100 ng) or a combination of CRH (500 ng), Fluoxetine (100 ng) and muscimol (10 or 50 ng). This experiment was run 3 times; January 1999 (6a), October (6b), and November (6c) 2000. Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($P > 0.05$, Dunn's multiple range test). $N = 20$ for all treatments in January and November. $N = 10$ for all treatments in October. Columns that have NT indicate that this treatment was not performed.

Experiment 7: Effect of bicuculline on serotonin/CRH induced hyperactivity

The effect of partial inhibition of the GABAergic system on the stimulatory effect of CRH and serotonin was evaluated. Fish were injected ICV with either saline, CRH (500ng), a combination of CRH (500 ng) and fluoxetine (100ng) or a combination of CRH (500 ng), fluoxetine (100 ng) and bicuculline (10 or 50 ng). Twenty fish were injected in each treatment group. The experiment was conducted over 2 days in November 2000. There was no difference in activity levels within any of the treatment groups over the two days; therefore, we combined data within each treatment. An ICV injection of CRH and fluoxetine stimulated locomotor activity ($P < 0.001$) (Fig 3). The concurrent administration of bicuculline (50 ng) with CRH and fluoxetine significantly potentiated this effect ($P < 0.05$).

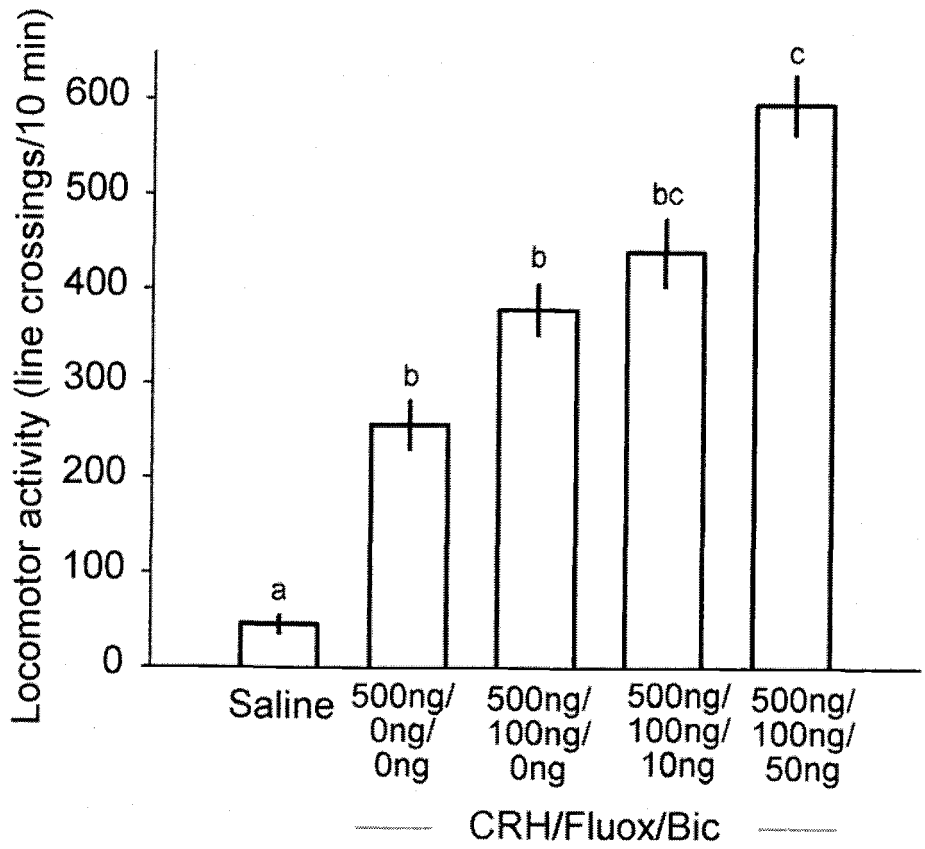

Figure V.7 Locomotor activity (mean ± 1 S.E.M.) in juvenile chinook salmon following ICV injections of saline, CRH (500 ng), a combination of CRH (500 ng) and fluoxetine (100 ng) or a combination of CRH (500 ng), fluoxetine (100 ng) and bicuculline (10 or 50 ng). Locomotor activity was measured as described in figure 1. Columns that share a common superscript are not different ($P > 0.05$, Dunn's multiple range test). $N = 20$ for all treatments.

Discussion

We have shown previously that endogenous GABAergic mechanisms are involved in the stimulation of locomotor activity in juvenile chinook salmon (Clements and Schreck, 2001a). In the current study we investigated the possibility that this effect was due to mediation of the dopaminergic system. Evangelista et al.

(1987) suggested that dopaminergic mechanisms may be involved in mediating the stimulatory effect of GABA on activity. Our results support this hypothesis, as administration of a dopamine receptor antagonist (haloperidol) significantly attenuated muscimol induced activity. Furthermore, the administration of a dopamine uptake inhibitor potentiated the effect of muscimol when given concurrently. It is not clear, however, whether the effect of muscimol was due to the direct or indirect action on dopamine releasing neurons. It is unlikely that non-specific binding of haloperidol to the GABA receptor is causing the attenuation in activity; GABA receptor binding studies indicate that haloperidol binds very weakly to GABA receptors (Christensen, et al. 1979). Similarly, it is unlikely that binding of GABA to dopamine receptors is responsible for the attenuation as muscimol has no effect on haloperidol binding or on dopamine sensitive adenylate cyclase (Evangelista et al. 1987). There is, however, evidence that muscimol or GABA can stimulate the release of dopamine from neurons (Giorguieff et al. 1978; Starr, 1978). Alternatively, it is possible that another intermediary pathway exists that is mediated by GABA. Interactions between serotonin and dopamine are complex and seem to be specific to certain substrates and behaviors. However, it appears that dopamine and serotonin often have mutually antagonistic effects (Bradford 1986), therefore an alternative hypothesis is that dopaminergic activity is favored because of the inhibition of serotonergic activity by muscimol.

In the current study fluoxetine clearly potentiated the effect of exogenous CRH on inducing hyperactivity. We also investigated a mechanism for downregulating

locomotor activity under the stimulation of CRH and 5-HT. Grossman et al. (1993) demonstrated that GABA inhibits CRH release within the hypothalamus *in vitro*. However, previous work in our laboratory indicated that GABA agonists and antagonists have no effect on locomotor activity induced by exogenous CRH alone (Clements and Schreck, 2001a). The administration of muscimol concurrently with CRH and fluoxetine produced mixed results. On one occasion muscimol attenuated the response to CRH and fluoxetine while on two later occasions muscimol had no effect. These differences may relate to the age of the fish. In the first experiment, fish were 14 months compared to those in experiments 2 and 3 that were nine months. The life history of these fish is such that we cannot rule out the possibility of seasonal changes in the responsiveness to various treatments. However, the response to other treatments is relatively homogeneous throughout the year so we are confident that these responses are generally applicable to this species. In contrast to the mixed results with muscimol, bicuculline clearly potentiated the combined effect of CRH and fluoxetine. Administration of haloperidol had no effect on activity levels induced by CRH in the current study. This suggests that haloperidol sensitive dopaminergic mechanisms do not play an important role in directly mediating the effects of CRH on locomotor activity. However, it may be interesting to determine the effect of DA agonists and antagonists on the interaction between CRH and 5-HT. Taken together these results suggest that endogenous GABA may indirectly regulate the effect of CRH on locomotor activity by inhibition of the serotonergic system.

In summary, this study provides evidence to support the hypothesis that endogenous GABA mediates dopaminergic mechanisms that are involved in the control of locomotor activity, possibly via disinhibition. Furthermore, our results suggest that GABA can indirectly inhibit the effect of CRH on locomotor activity by affecting the serotonergic system. Future work that focuses on determining how specific environmental cues such as the presence of predators, toxins, food or more subtle cues such as changes in daylength, are processed to produce a specific locomotor response (hiding, feeding, migration) would represent a great advance in our understanding of how fish are likely to respond to environmental changes as a result of human activity. The current results provide a framework for our understanding of the neural systems that are involved in behavioral control at a macroscopic level, however, isolation of specific CNS substrates is required to make further advances.

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CHAPTER VI

CENTRAL ADMINISTRATION OF CORTICOTROPIN-RELEASING HORMONE ALTERS MIGRATORY BEHAVIOR IN JUVENILE CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*).

Shaun Clements and Carl B. Schreck

Oregon Cooperative Fish and Wildlife Research Unit[2], Department of Fisheries and Wildlife and U.S.G.S (for C.B.S), Oregon State University, Corvallis, OR 97331-3803, USA.

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Abstract

We evaluated the effect of corticotropin-releasing hormone (CRH) on downstream swimming behavior in wild and hatchery juvenile chinook salmon (*Oncorhynchus tshawytscha*). To determine whether CRH is involved in initiating downstream movement, fish were given an intracerebroventricular (ICV) injection of either saline or CRH (500ng). Following a short recovery period the fish were released into the middle of an artificial stream system. CRH significantly increased the proportion of fish that were distributed downstream of the release site.

To test the effect of CRH on downstream swimming speed fish were given ICV injections of saline or CRH (500 ng) and released near the top of the stream. The time taken to pass through the system was recorded. Administration of CRH resulted in a bimodal response. In all cases the groups given ICV CRH had a higher proportion of fish that did not pass through the lower end of the system within 60 min of release. However, in those fish that did swim past this point, treatment with CRH increased the speed of downstream movement relative to saline injected controls.

Fall chinook that were migrating in the Elk River were given ICV injections of saline or CRH (500 ng) either 2, 3, or 7 days after transport from the river. The responses were similar to hatchery fish as a significantly higher proportion of fish that were administered CRH did not move completely through the system. However, the behavior of wild fall chinook that were injected with saline changed over time. The mean time for these fish to pass through the stream system

decreased on each successive day (day 2 > day 3 > day 7). In contrast, the behavior of the wild fish that were given CRH was relatively constant through time. These fish migrated more quickly than wild fish given ICV saline 40 h after transport from the river. However, 3 and 7 days after transport there was no difference in migration rate between the two treatments.

These results support the hypothesis that endogenous CRH within the central nervous system (CNS) is involved in the stimulation of downstream swimming behavior in juvenile salmonids. Furthermore, we speculate that CRH may also alter migratory behavior as part of the response to a stressor.

Introduction

The downstream migration of juvenile salmonids is a critical life history stage, during which significant physiological, morphological and behavioral changes occur to prepare the fish for life in seawater. This is referred to as the parr-smolt transformation (Hoar, 1963). We currently have a general understanding of these physiological changes that include increases in plasma thyroxine and cortisol (Folmar and Dickhoff, 1981; Specker and Schreck, 1982). Behavioral changes may include a decrease in territoriality and aggression and the subsequent formation of aggregations for the downstream migration (Iwata et al. 1990). However, the neuroendocrine systems initiating and controlling migratory behavior are poorly understood. Hormones of the adrenal/interrenal axis can regulate locomotor activity in mammals (Sutton et al. 1982), amphibians (Moore et al. 1984), and teleosts

(Castonguay and Cyr, 1998; Clements et al. 2001b). Many of these hormones regulate both behavior and physiology. For example, corticotropin-releasing hormone (CRH) is the initiating hormone in the response of the hypothalamic-pituitary-interrenal (HPI) axis to stress. It stimulates secretion of ACTH from the pituitary, thereby regulating the peripheral responses to stress. CRH is also involved in the control of many behaviors, particularly those that are expressed during the stress response (Butler et al. 1990; Takahashi et al. 1989; To et al. 1999).

During the smoltification process, as a consequence of adapting for a hypersaline environment, fish become maladapted for life in freshwater. Although there is debate about the absolute timing of these changes, one hypothesis is that this maladaptation forces fish to migrate in order to avoid deleterious effects on growth and survival (Thorpe, 1984; Thorpe, 1994). By extension, such a hypothesis implies that the freshwater environment represents a stressor that is avoided by migration to seawater. Indeed, one of the more predictable changes in the physiology of these fish is a pronounced increase in basal levels of cortisol, a primary indicator of the stress response (Specker and Schreck, 1982). Based on these observations, our previous work with salmonids, and the central role of CRH in the behavioral and physiological response to stressors, we hypothesized a duel role for endogenous CRH; that it might be involved in the initiation of migratory behavior, but also, that at high levels following exposure to a stressor, it might disrupt migratory patterns. The objective of this study is to test whether (1) CRH induces downstream swimming behavior in juvenile salmonids outside the normal

period of migratory movement, and (2) CRH would alter downstream swimming behavior in hatchery fish at a time when they are expected to migrate and in wild juveniles that were caught during the outmigration.

General Methods

Fish

Juvenile spring chinook salmon parr (*Oncorhynchus tshawytscha*) (Willamette stock), were raised under ambient photoperiod in a 336 L circular tank at Oregon State University's Fish Performance and Genetics Laboratory. Flow through water (12^0C) was supplied from a well. Fish were fed twice daily with semi-moist pellet (BioOregon™). The fish in experiment 1 were 11 months old and 115 ± 0.6 mm in length. The fish in experiment 2 were 8 months old and 109 ± 0.7 mm in length. Wild fall chinook were obtained from a screw trap on the Elk River in Southern Oregon. These fish were 0+ migrants (length: 55 ± 0.7 mm).

Chemicals

Ovine CRH was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in teleost Ringers solution (0.2 % $NaHCO_3$ in 0.6 % NaCl solution). At the beginning of each study, preparations of the appropriate concentration was made by serial dilution of the stock in the Ringers solution. A fresh preparation was used on each day of testing. Both the stock solutions and the daily preparations were stored frozen (-20^0C) when not in use.

Behavioral tests

The tests were conducted in artificial streams at the Forest Science Laboratory, Corvallis, OR. Two streams were used for these experiments; one for acclimating and holding fish prior to the experiments and the second for running the trials. The design of the stream channels is described in detail by Reeves et al. (1983) and illustrated in figure VI.1. Briefly, the streams were oval in shape with outside dimensions of 4.27 m x 4.88 m. Each channel was 0.77 m wide and 0.6 m deep. The total volume of each stream was 5866 L. The channels were filled with sand and rocks of varying sizes that were placed to form several pools, runs, and riffles. The inner wall of the stream was constructed of 6.35 mm plexiglass to enable an observer to view the fish in the stream. Black polyethylene curtains were suspended on either side of the channels to minimize visual disturbance. The lighting system was electronically controlled and was designed to mimic the sunrise-day-sunset-night cycle. Each channel was lit by nine 60-watt incandescent bulbs with 12 inch reflectors. The flow was set-up by a paddle wheel that produced a current of approximately 0.46 m sec^{-1}. Prior to the experiments the streams were filled with dechlorinated water, which was then chilled to $13^{0}C$. Two screens were installed on either side of the paddle wheel to prevent fish from entering this area. A trap box was installed immediately above the lower screen and consisted of a plexiglass box with a narrow V opening to allow the passage of fish into the holding area between the trap and the screen. A mesh fence was placed above the trap box to guide fish into the mouth of the trap.

Fish were transferred from the hatchery (30 min) or the river (5 h) to 1 of 3 holding areas in the lower artificial stream on day 0. All fish were given at least 40 h to facilitate acclimation to the stream environment. For all experiments there were 2 treatment groups. Each replicate (n=10) was netted from the holding areas using a stratified random design and placed in anesthetic. Fish were then injected with either 1 μL Ringers solution or 500 ng CRH (1 μL). Following the injection all the fish in a replicate were transferred to the stream channel and placed in a dark perforated plastic container for recovery. Twenty min later the container was removed and the fish were able to swim freely. To account for possible diurnal variations in behavior the treatments were alternated throughout the day.

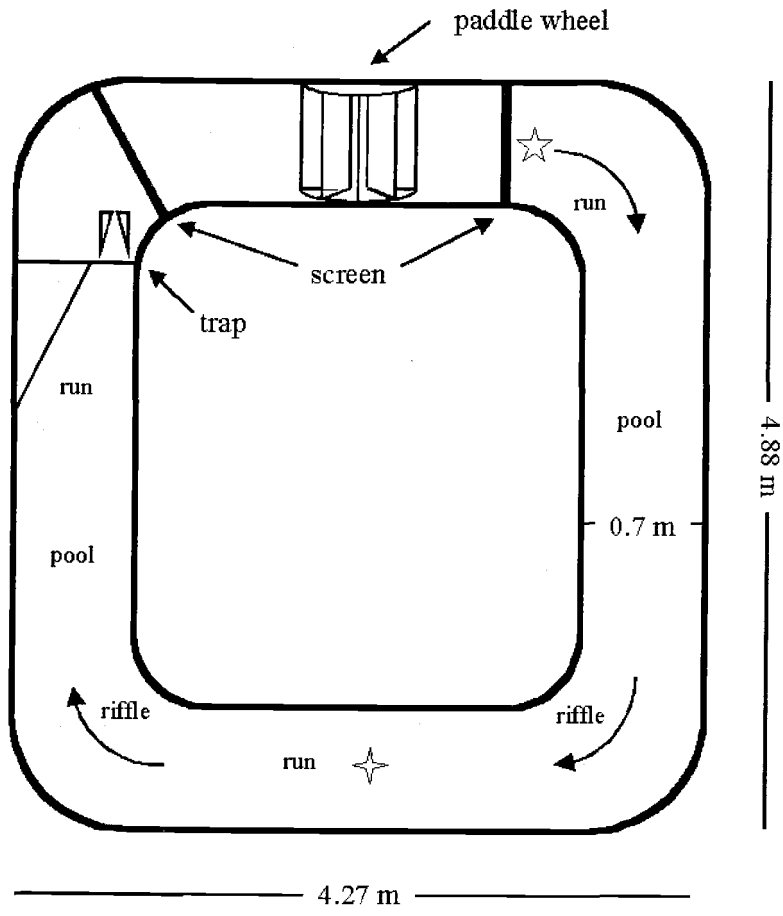

Figure VI.1 Illustration of the artificial stream system used in the current study. Fish were released in the middle of the stream in experiment 1.✧ Fish were released at the upper end of the stream in experiments 2 and 3☆

Administration procedure

Fish were netted from the holding area and placed in anesthetic until they lost equilibrium (5-7 min in 50 mg L^{-1} tricaine methanesulfonate buffered with 125 mg L^{-1} $NaHCO_3$). Chemicals were administered freehand under a dissecting microscope using a 10 μL glass syringe with a 26 G needle (Hamilton # 701). In

preliminary trials this was found to be more accurate than using a stereotactic micrometer. Injections were performed midline, immediately behind the pineal gland to a depth of 1-2 mm below the tissue surface. To prevent excess leakage of the chemicals, the 1 μL volume was infused slowly and the needle was held in position for 5 s after the infusion to allow for clearance. The injection procedure took between 15-20 s per fish. The syringe and needle were rinsed with Ringers solution between fish and were cleaned with ethanol and Ringers following each day's testing. In preliminary studies, injections with a dye tracer confirmed that the solution was being injected into the third ventricle, with > 85% accuracy.

Statistical Analysis

In experiment 1 the data was expressed as the distance from the release point. Positive values indicated the fish was downstream while negative values were assigned to fish that were above the release point. Differences between replicates and treatments were analyzed using a Kruskal-Wallis test. In experiments 2 and 3 the effect of day and replicate on the percentage of fish that migrated to the lower end of the stream were analyzed using the Breslow Day (B-D) Homogeneity of Odds Ratio test. Treatment effects were analyzed using the exact Cochran Mantel Haenszel test (SAS v 7.0). During the second part of the analysis for experiments 2 and 3 only those fish that moved completely through the system were considered. The data for travel time to the end of the stream system were transformed to ranks

because of unequal variances. Day, replicate, treatment and interaction effects were analyzed using ANOVA on the transformed data (Statgraphics Plus v 4.0).

Specific Methods and Results

Experiment 1

To determine if CRH induces downstream swimming behavior, hatchery spring chinook were injected with either saline or CRH. The fish were released at the midpoint in the stream (Fig 1). Five, 10, and 15 min after the fish were liberated, their position in the stream was recorded as the distance up or downstream from the release site. The experiment was repeated 4 times for each treatment (Saline, N = 40; CRH, N = 39). The experiment was conducted on November 3 1999.

There were no differences in the distribution of fish between replicates within a treatment therefore the data were pooled. For both treatments the fish tended to move downstream (Fig. VI.2).

However fish that were injected with CRH were distributed significantly further downstream from the release point than fish that were administered saline (5 min, P = 0.000; 10 min, P =0.000; 15 min, P = 0.001) (Fig. VI.3). For both treatments there was no change in the average distance from the release point over time.

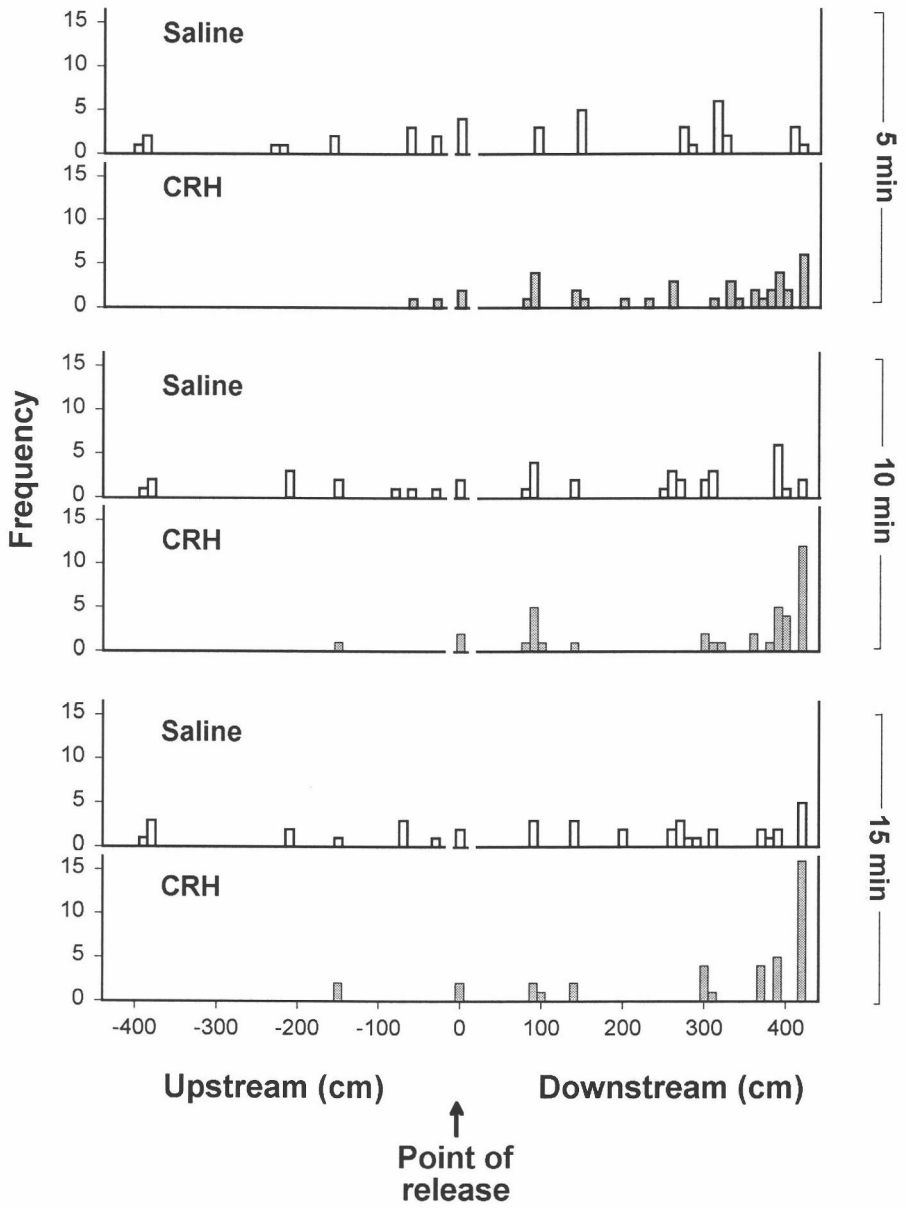

Figure VI.2 Frequency distribution of the distance up and downstream from the release point in juvenile hatchery spring chinook salmon following ICV injections of saline or CRH (500 ng). Positional data was recorded 5, 10 and 15 min post release.

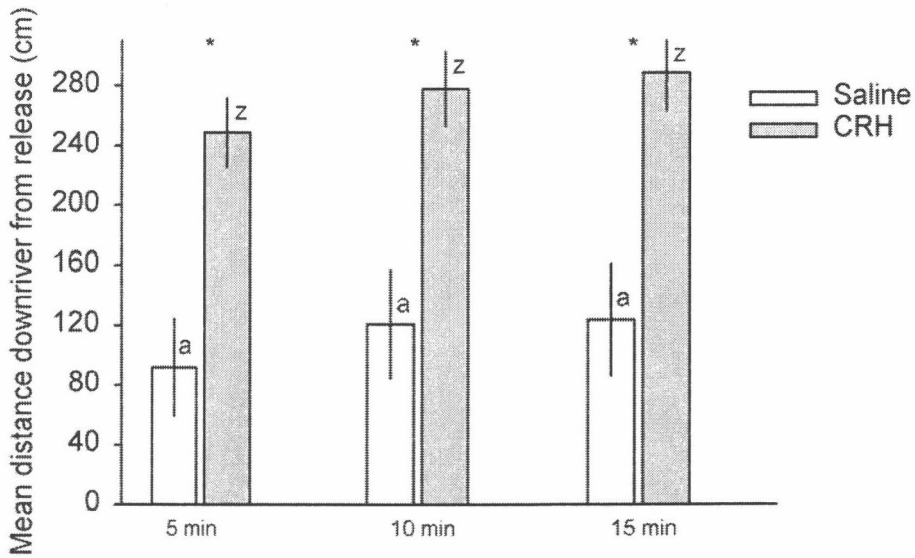

Figure VI.3 Mean (± SEM) distance downstream from the release point 5, 10 and 15 min after release following an ICV injection of saline or CRH (500 ng). N = 40 for saline; N = 39 for CRH. Columns within a treatment that share a common superscript are not different ($P > 0.05$, ANOVA). * indicates a significant difference between treatments ($P < 0.05$ ANOVA).

Experiment 2

The effect of ICV administration of CRH on downstream swimming behavior in hatchery fish was evaluated. Fish were injected with either saline or CRH and released into the stream near the upper end of the channel (Fig. 1). An 8 mm video camera was mounted in front of the trap at the lower end of the stream to record fish as they entered the trap. Migration speed was calculated as the time between release and entry to the trap. The experiment was conducted over 2 consecutive days in July 1999 beginning 2 days after fish were transported to the facility. Three replicates of each treatment were tested on each day (Saline, N = 59; CRH, N = 57).

There was no effect of day or replicate on the percentage of fish that migrated ($P = 0.538$, B-D homogeneity test). ICV injections of CRH significantly increased the proportion of fish that did not pass through the lower end of the system ($P < 0.001$, CHMT) (Fig VI.4).

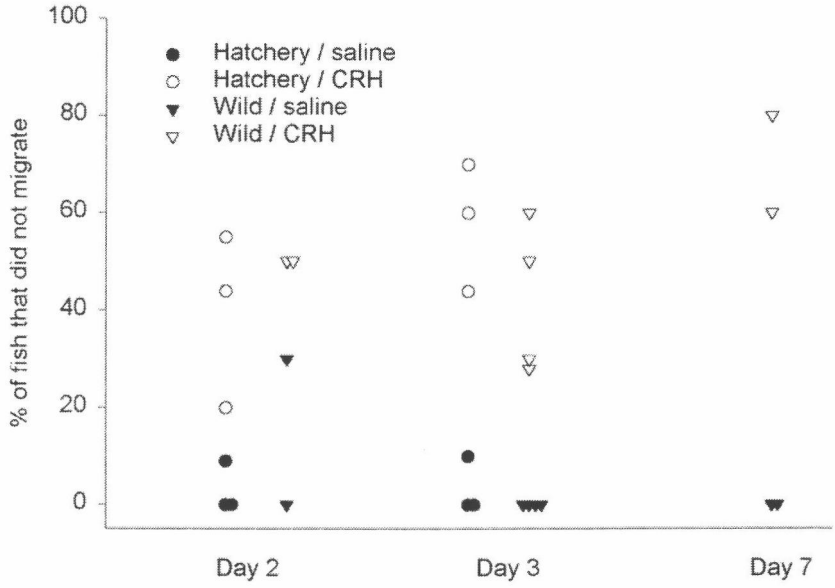

Figure VI.4 Pecentage of fish that did not migrate through an artificial stream system following an ICV injection of saline of CRH. Each point represent the proportion of fish from a single replicate. On all days and for both hatchery and wild fish, treatment with CRH resulted in a significantly greater proportion of fish that did not migrate through the system ($P < 0.05$; CHMT exact test).

Of the fish that did enter the trap there was no difference in the travel time between the replicates for either treatment (saline, $P = 0.55$; CRH, $P = 0.34$). Similarly, there were no differences in mean travel time between days for either

treatment (saline, P = 0.44; CRH, P = 0.12). Therefore, the data were combined for further analysis.

An ICV injection of CRH significantly decreased the mean travel time compared to fish that were given a sham injection (P = 0.000) (Fig. VI.4). Travel times for fish given CRH were 13.87 ± 2.01 min (mean ± 1 SEM), while for fish given saline they were 24 .43 ± 0.56 min.

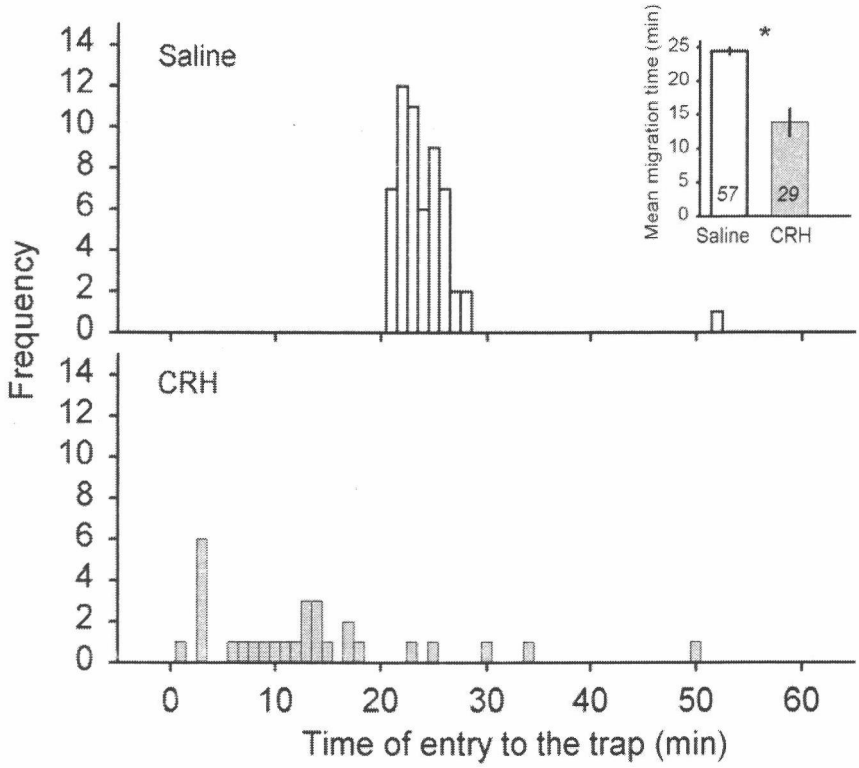

Figure VI.5 Frequency distribution of the time to migrate through an artificial stream system by juvenile hatchery spring chinook salmon following ICV injections of saline or CRH (500 ng). Migration times were calculated as the time from release until the time of entry to a trap at the downstream end of the stream. The inset shows the mean (± SEM) migration time for fish in all 6 replicates. *N* values are given in italics. * indicates a significant difference between treatments ($P < 0.05$ ANOVA).

Experiment 3

The effect of ICV administration of CRH on downstream swimming behavior in wild, migrating fish was evaluated. The experimental setup was identical to Experiment 1. However, the experiment was conducted on 3 days in August 1999 (2, 3, and 7 days after fish were transported to the facility). On days 2 and 7, two replicates of each treatment were tested whereas on day 3, four replicates of each treatment were tested (Saline, $N = 80$; CRH, $N = 80$).

There was no effect of day or replicate on the percentage of fish that migrated ($P = 0.108$, B-D homogeneity test). ICV injections of CRH significantly increased the proportion of fish that did not pass through the lower end of the system ($P < 0.001$, CHMT) (Fig VI.4).

The frequency of fish migrating to the end of the system over time is illustrated in Fig 5. Of the fish that did enter the trap there were differences in mean travel time between days for fish that were given the sham injection treatment ($P = 0.000$), but not for fish given ICV CRH ($P = 0.397$) (Fig VI.6). Therefore, the data were analyzed separately for each day. Data from each replicate were combined within a treatment because there were no significant differences.

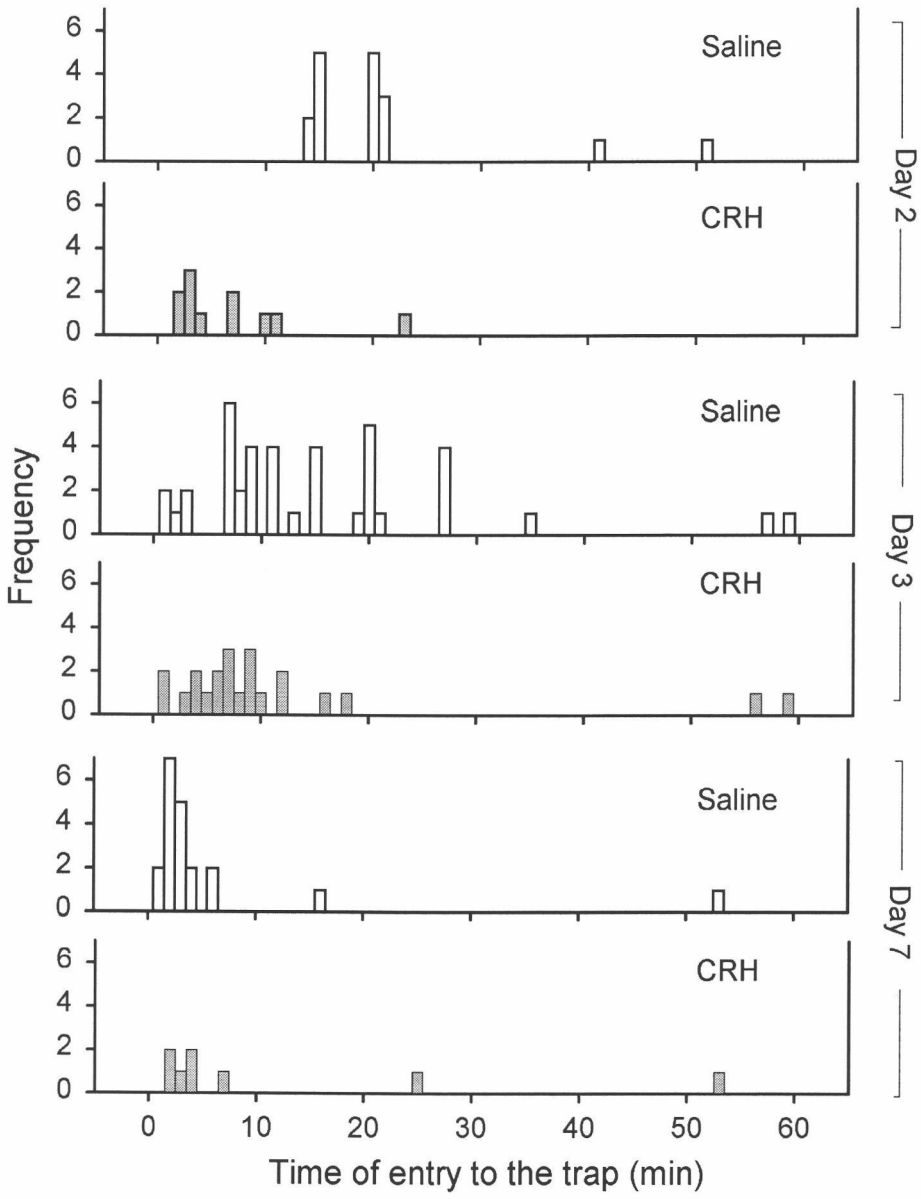

Figure VI.6 Frequency distribution of the time to migrate through an artificial stream system in juvenile wild fall chinook salmon following ICV injections of saline or CRH (500 ng). The distribution is presented separately for each day (2, 3, and 7) of the experiment. Migration times were calculated as the time from release until the time of entry to a trap at the downstream end of the stream.

Fish that were administered saline moved downstream significantly faster on

each successive day ($P < 0.05$). An ICV injection of CRH significantly decreased the travel time compared to fish that were given a sham injection on day 2 ($P = 0.005$), but not on days 3 and 7 (Fig VI.7).

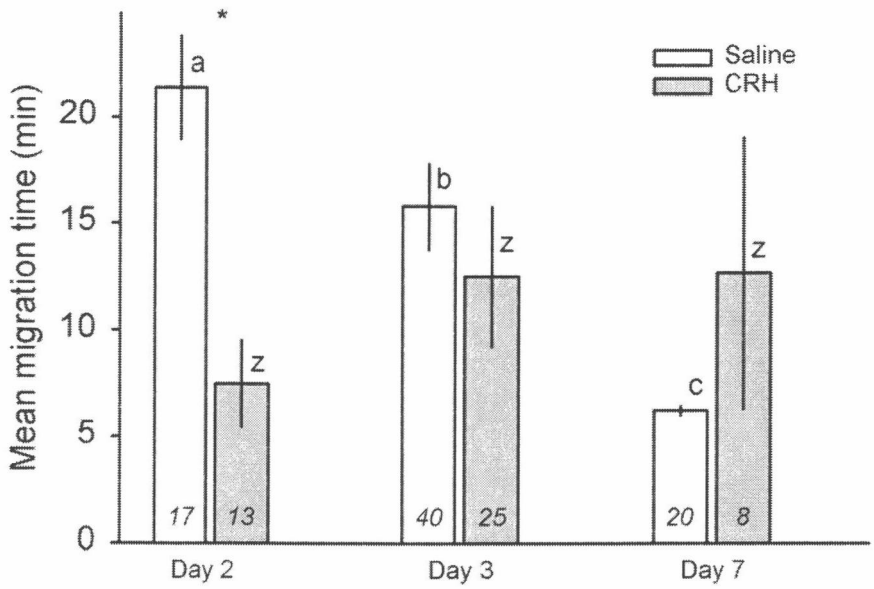

Figure VI.7 The effect of CRH on migration rates in wild fall chinook. Fish were given ICV injections of saline or CRH (500ng). Each column represents the mean (± SEM) migration time (min) through an artificial stream system of 2-4 replicates. Total *N* values are given in italics. Columns within a treatment that share a common superscript are not different ($P > 0.05$, ANOVA). * indicates a significant difference between treatments ($P < 0.05$ ANOVA).

Discussion

The results of these experiments provide clear evidence that the brain hormone CRH can alter the downstream swimming behavior of juvenile chinook salmon in a simulated stream environment. Furthermore, they are consistent with the hypothesis that endogenous CRH is involved in the both the initiation of migratory behavior

by increasing the tendancy to move downstream; and once the fish is migrating, modifying downstream swimming behavior in response to stressors. The results of the experiment examining the distribution of fish following ICV treatment with CRH show that CRH increases the tendency to move downstream. Fish treated with saline were distributed approximately 40/60 upstream/downstream of the point of release. At the time of the experiment these fish were parr, therefore such a distribution would be expected. Treatment with CRH, however, resulted in a distribution that was highly skewed to the downstream end of the stream (5/95). This result suggests that CRH promotes downstream orientation in these fish.

Earlier in the season in experiment 2, when a number of our hatchery fish are smolting, the behavior of the fish that were injected with saline was very homogeneous. In general the majority of these fish tended to migrate through the system at similar rates to each other. In contrast, ICV injections of CRH dramatically altered the behavior. Almost half the fish did not migrate through the system, compared to 3% of fish treated with saline. It is not clear whether this represents holding behavior or increased non-directional movements. Of those fish that did complete the course however, the time to completion was significantly faster than saline treated fish. The reasons for the bimodal behavior following injections of CRH are not clear. However, it is not uncommon for animals to exhibit a fight or flight response to a stressor. By delaying the migration the fish may either be attempting to hasten recovery or exhibiting disorientation from the stressor. Alternatively, those fish that moved faster may be attempting to move

away from the source of the stressor, and it is also possible that these fish would have delayed their downstream movement at a later time.

The behavior of the fish that were caught during the outmigration was relatively similar to those that were taken from the hatchery at the same time. However, the shift in the behavior of those fish from the Elk River that were administered saline is of particular interest. We suggest that this may be due to an increase in what we would refer to as 'frustration' from being held. So that when these fish are able to move downstream they do so at a greater rate than fish that have not been held for as long. It is widely recognized that in mammals CRH promotes behaviors associated with increased emotionality or sensitivity to stress (Britton et al. 1986 a,b; Butler et al. 1990; Koob and Britton, 1990). As yet we are lacking tests to determine if fish experience these phenomena and, if so, whether CRH mediates such behaviors. However, the migratory urge is very powerful, therefore any disruption is likely to represent a stress to the fish. An alternate hypothesis is that the transportation process resulted in a stress response that initially inhibited the downstream movement of these fish.

It is generally accepted that CRH can have direct neurotropic actions, independent of the hypophyseal system (Sutton et al. 1982; Moore et al. 1984; Clements and Schreck, 2001). However, CRH also operates as part of a complex system of hormones and neurotransmitters, many of which can potentially alter behaviors such as locomotor activity. For example a number of experiments suggest that thyroid hormones within the central nervous system (CNS) may also

affect locomotor behavior in fishes (Hoar et al. 1952; Hoar et al. 1955, Sage, 1968; Iwata 1995). Thyroid hormones also play an integral role in regulating a number of processes during smoltification Langdon (1985). Based on these observations a possible role for thyroid hormones in the control of migratory behavior has been proposed. One possible hypothesis for the control of such a system would involve CRH. Larsen et al. (1998) showed evidence that CRH may be involved in regulating the hypothalamic-pituitary-thyroid axis in fish by demonstrating that CRH induces the secretion of thyrotropin *in vitro.*

The final expression of migratory behavior, however, is likely to be a result of the integration of many signals. A system that can simultaneously mediate both physiological and behavioral changes, and one that is responsive to external stressors is clearly of value. Many lines of evidence from multiple studies support the hypothesis that CRH may be central to such a system. CRH appears to increase temporally during the smoltification period in discrete brain regions (Ebbesson pers. comm.). CRH is involved in the regulation of many neurotransmitter systems, that in turn regulate a variety of behaviors (Lowry et al. 2000; Price and Lucki, 2001; Clements et al. submitted). In the current study there is evidence that CRH produces behavioral effects that mimic those of fish that are stressed during their downstream migration. Similarly, ICV administration of CRH increased the tendency of juvenile salmonids to move downstream. Finally, as mentioned earlier, CRH can regulate the activity of the HPT axis (Larsen et al. 1998), which is involved in producing many of the morphological, physiological, and behavioral

changes observed during smolting.

How such a system that controls migration might be environmentally regulated is speculative at best. Many studies have shown that day length is a potent cue for smoltification and migration (Berg et al. 1994; Randall et al. 1994). Vertebrates transduce this signal by production of melatonin, during the hours of darkness. Previous work suggests that melatonin alters the synthesis or release of CRH (Jones et al. 1976; Esquifino et al. 1997). Based on these observations we hypothesize that this link provides a starting point for an examination of the relationship between the environment, physiology and migratory behavior.

This research has implications for the decline of several populations of anadromous fish in the Pacific Northwest of the USA. There is considerable evidence suggesting that disruption to migratory pathways of both juveniles and adults is contributing to this decline. A large number of man made obstacles are in place on rivers in this region, including hydroelectric and irrigation dams and passage through such structures can induce a severe stress response in juvenile salmonids (Congleton et al. 2000). Studies of fish behavior in the wild suggest that stress can disrupt the downstream migration, and often leads to fish holding for extended periods in areas of high predation (Clugston and Schreck, 1992; Snelling and Schreck, 1993). Our results suggest that the disruption in the downstream migration following exposure to a stressor may be mediated by CRH. However, it is not clear what effect any disruption has on subsequent performance and ultimately survival. Potentially the juvenile salmonids could be exposed to

predators for a longer period while they recover from the stressor.

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CHAPTER VII

CONCLUDING REMARKS

The main goal of this thesis was to identify the central (brain) mechanisms involved in the control of locomotor activity in juvenile salmonids. This was examined by manipulations of 3 neurotransmitter systems; serotonin (5-HT), dopamine (DA), γ-amino-n-butyric acid (GABA) and the neuropeptide corticotropin-releasing hormone (CRH). Behavioral and physiological assays were used to determine the effect of these manipulations.

The results suggest that all 4 systems play some role in the regulation of locomotor activity. However, the findings presented in this thesis only partially suggest the complexity of the brain and its control of locomotor behavior. Indeed, the literature suggests that many more hormones, neurohormones, and neurotransmitters can alter activity in vertebrates (e.g. Winberg et al. 1993; Osborne, 1994; Kemnitz et al. 1995). Furthermore, these systems often have multiple layers of interactions and there is evidence that the nature and sensitivity of many of the systems change over time.

To aid the understanding of this thesis I have proposed a simple model (Fig. VII.1). The model is based on whole animal studies that are conducted in this thesis, with support from the literature. This model is intended to represent the likely effects of gross changes in brain chemistry, rather than fine scale changes in

subpopulations of neurons. However, it does provide a framework to understand the results that have been presented and for proposing future research.

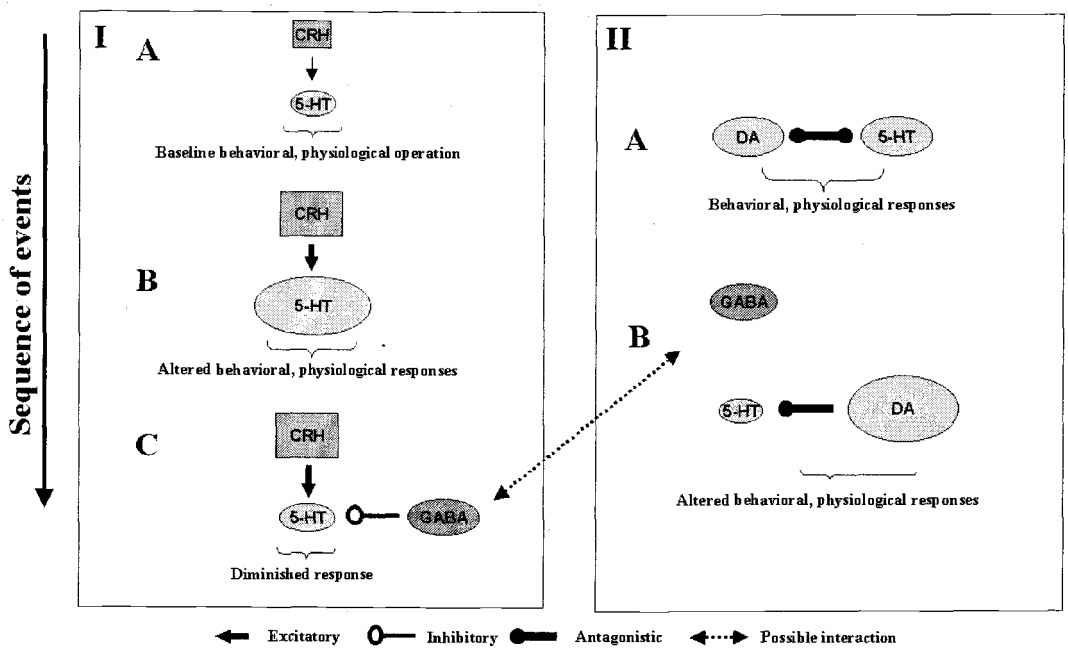

Fig. VII 1. Proposed model of the interactions between CRH, serotonin (5-HT), GABA, and dopamine (DA). The size of each box indicates the relative activity of that system. Panel I shows a sequence of events involving CRH, serotonin and GABA. (A) baseline levels of CRH and serotonin maintain some level of activity. (B) A stimulus is received to increase production of CRH. This mediates an increase in the production of 5-HT which then mediates behavior such as an increase in locomotor activity. (C) Negative control occurs when levels of endogenous GABA rise inhibiting the action of 5-HT and decreasing the behavioral/physiological output. Panel II illustrates the hypothetical disinhibition of the dopaminergic system by endogenous GABA. (A) The interaction between 5-HT and DA determines the final behavioral output. (B) If serotonergic cells are targeted by endogenous GABA the inhibition of DA by serotonin will be removed, allowing DA mediated behaviors to predominate.

In the current study, I chose the third ventricle to administer chemicals because of its proximity to areas that might be involved in locomotor control. Given the paucity of information on where this control occurs in teleosts, the best strategy was to choose a site that allowed stimulation of a large area of the brain. The third ventricle is located in the diencephalon but continues into the mesencephalon via the aqueduct of Sylvius (Takashima and Hibiya, 1995). Injections into the third ventricle are therefore most likely to affect the areas of the diencephalon, including the preoptic nucleus, nucleus preopticus magnocelluraris, nucleus preopticus parvocellularis. It is also possible that diffusion into the thalamus, and other regions of the hypothalamus occurs. However the most likely site for causing the behavioral effects observed in this study is the mesencephalon. This region contains the integration centers for the visual and other senses and locomotion.

By injecting CRH into the 3rd ventricle I demonstrated that CRH causes an increase in locomotor activity. Further studies showed that this effect is potentiated by the serotonergic system. There is evidence from other studies that the synergistic effect is due to the stimulation of serotonergic fibers by the release of CRH (Lazosky and Britten, 1991; Singh et al. 1992; Lowry et al. 2000). However, an alternate hypothesis is that the release of CRH is stimulated by serotonin. A number of studies, both *in vitro* and *in vivo,* have shown that this occurs in the vertebrate brain (Jones et al. 1976; Tizabi and Calogero, 1992). If this was the mechanism, I would have expected that injections of fluoxetine alone would cause an increase in activity in the current studies. However, this was not observed;

therefore our results tend to support the initial hypothesis that the increase in locomotor activity is due to the stimulation of the serotonergic system by CRH.

The negative control of many neural pathways is by the action of neurotransmitters such as GABA and glycine. I evaluated whether GABA exerts negative control on CRH or the combination of CRH and serotonin. The results suggested that GABA is only inhibitory of the CRH/serotonin combination and not CRH alone, implying that GABA exerts its effect on serotonin. However, a number of *in vitro* experiments suggest that GABA can directly inhibit the release of CRH (Jones, et al 1976, Calogero et al. 1988a,b; Tizabi and Calogero, 1992). Therefore, it is also possible that the current experimental design was not able to detect such an effect. If this is the case, it is most likely because the system was already stimulated by the addition of exogenous CRH so that any effect of GABA on the release of endogenous CRH would be masked. The issue was further confounded as the administration of GABA alone induced an increase in activity. I later hypothesized that this was due to the disinhibition of the dopaminergic system. Our results showed that the stimulatory effect of muscimol on locomotor activity was attenuated when the dopaminergic system was inhibited. Similarly, the effect of GABA and dopamine on activity was additive. What was not immediately clear from these studies was how GABA interacts with the dopaminergic system. Given that GABA appears to have some inhibitory effect on serotonin, I have proposed the hypothesis that by inhibiting serotonin, dopaminergic activity is increased. This

is supported by studies that show that in many regions of the brain serotonin and dopamine exert mutually antagonistic effects (Bradford 1986).

One of the biggest hurdles to comprehending the results that suggest interactions between the chemicals is understanding the direction of control. In particular, evidence is emerging that the effect of a specific chemical varies depending on which subpopulation of cells is the target (Lowry et al. 2000). This kind of precise control within the brain, and regional and temporal variability in the connection of neurons means that future studies specifically targeting the brain region of interest will be invaluable to the advancement of the work in this thesis. The current work provides some evidence for these links, but it will not be until many lines of convergent evidence point in the same direction that we will be able to confirm these relationships. Techniques such as neuronal tract tracing and *in-situ* hybridization are both tools that could be used in addition to the behavioral assays.

These results most likely have a broad field of applicability amongst vertebrates. The same neuroactive substances that were tested in this thesis are present in all vertebrates, and furthermore, it appears that many of the effects are similar. However, I have also investigated whether CRH is involved in mediating specific processes in juvenile salmonids. In particular, the control of downstream migration in juvenile salmonids was of interest. In chapter 5 I tested the hypothesis that CRH was involved both in the initiation of migration and in modifying behavior during the migration, possibly as part of the stress response. Thorpe (1984) proposed the notion that the downstream migration of juvenile salmonids is

an avoidance response to maladaption in the freshwater environment. Various lines of evidence support the hypothesis that CRH could potentially initiate migration as part of this avoidance response. In support, my results showed that injections of CRH increased the tendancy of non-migratory fish to move downstream. However, we have also noticed that, once migrating, salmonids are susceptible to disruptions in migratory behavior following exposure to a stressor. Therefore, I also hypothesized that once fish were already migrating exposure to a stressor would disrupt migratory behavior via the action of CRH. Fish could 'choose' to either: move away from the stressor; cease movement and hide from the stressor; or, cease movement and recover from the stressor. The final choice is likely to be affected by: the prior experience of the fish; the internal state, for instance energy levels; inherited traits, that may include the innate capacity to respond to a stresssor; and the physical characteristics of the environment. For instance, if there are places to hide the fish might hold in these areas or, if it is an open environment the fish may move more quickly to get through the area. These factors are illustrated in Fig VII 2. The results presented in this thesis provide the first clues that CRH may play a role in determining migratory behavior and provide support for the early hypotheses. I showed that CRH increased the tendency of fish to move downstream. This work will be continued by examining the temporal changes in CRH during the smolting period either using suppression subtractive hybridization or *in-situ* hybridization.

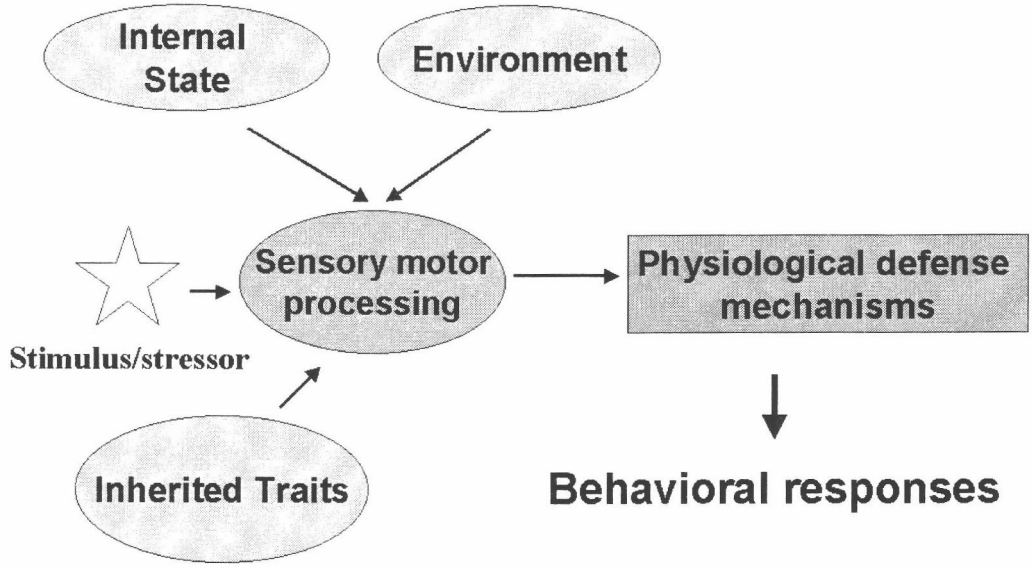

Fig. VII 2. Theoretical model of the process that occurs following exposure to a stressor and leading to behavioral changes. The stimulus is processed in the brain taking into account factors such as the internal state (e.g. energy levels, or brain chemical levels); the environment (whether there are places to hide); and inherited traits (such as the innate capacity to respond to a stressor). When these signals are processed a response is mounted that may include increases in catecholamines to mobilize energy, or changes in neurotransmission. Changes in the physiology then lead to alterations in the behavior.

If CRH is involved in the initiation of migratory behavior, the next step would be to identify how the environment is involved in mediating this effect. I have discussed the possibility that the migration is purely an avoidance response as the fish become maladapted to freshwater. In this scenario CRH would function as part of the stress response. In chapter 5 I discussed an alternative hypothesis that changes in the diel cycle cue the migration by altering the pattern of melatonin production, and that melatonin then regulates the production of CRH. This might

occur directly, as there is some limited evidence suggesting that melatonin modifies the output of CRH (Jones et al. 1976; Esquifino et al. 1997). Alternatively, the effect may be indirect, as there is also evidence that melatonin can modify the output of serotonin (Cardinali, 1975; Chuang et al. 1993), which could then lead to changes in CRH (Jones et al. 1976). This is supported by a number of studies showing that serotonin stimulates the release of CRH (Jones et al. 1976; Tizabi and Calogero, 1992). What is less clear, perhaps, is whether such a system would be transient, with the formation of neuronal networks to mediate the change from a resident to a migrant fish during the smoltification period. Or, if permanent, then the question remains: what part of the environmental signal would the system respond to, an absolute level or the rate of change? Recently, it was reported that a specific population of CRH-like neurons appeared transiently around the time of smolting (Ebbesson pers. comm.). It would be of great interest to determine the role of these neurons, specifically whether they are involved in mediating migratory behavior.

In addition to it's hypothesized effects on migration per se. there is evidence that CRH may be involved in mediating other behaviors associated with the downstream migration of juvenile salmonids. Sociality is important among chinook salmon in particular. Kelsey (1997) reported that juvenile chinook salmon normally show a high degree of shoaling behavior, evidenced, in part, by low inter-fish spacing, and very little aggression between fish. Similarly the downstream migration is characterized by a shift from territoriality and aggression based

behaviors to a more gregarious, social expression. Previous studies have shown that the administration of CRH alters social and exploratory behaviors in mammals (Kalivas et al. 1987; Winslow et al. 1989; Monnikes et al. 1992). In chapter 2, I showed that CRH decreases the contact time with conspecifics. However, it is not clear if this effect relates to a choice to avoid other fish or simply to the increase in activity. It would also be interesting to determine if CRH has any effects on the shoaling behavior exhibited during the downstream migration of juvenile salmonids. In chapters 2 and 3 I showed that both CRH and serotonin altered habitat choice. In general control fish tended to spend the majority of their time near the sides of the tanks. Treatment with CRH or with fluoxetine significantly increased the amount of time fish spent near the center of the tanks. For fluoxetine, at least, this effect is not due to an increase in activity, as these fish had significantly lower levels of locomotor activity than control fish. Such changes in habitat choice also occur during the smoltification process as fish move from the stream bottoms to adopt a more pelagic life.

An underlying theme to this research was to lay a foundation for determining the physiological basis by which stress alters behaviors. This is particularly important given that in many regions wild stocks are threatened by human activities. Part of the problem in such cases may be the activation of the stress response so that critical functions such as development (e.g. smolification), migration, or reproduction are delayed, inhibited or advanced. By understanding how the physiology affects behavior during stress it may be possible to manage the

environment so that the fish can better cope with and survive the challenge. Although this is always a difficult proposition, the following example serves to illustrate how this might work at a fish passage barrier. Passage through such structures is stressful, and field studies suggest that fish often delay their downstream migration following exposure to such a stressor and hold thus making them more vulnerable to predators (Clugston and Schreck, 1992; Snelling and Schreck, 1993). If the flow through the structure was managed in such a way that the majority of fish were routed past areas of slow water and potentially high numbers of predators then survival could be enhanced.

Although these studies were not designed to show a direct link between the effects of stress on behavior and CRH. There is sufficient evidence in the literature to suggest that CRH is heavily involved in mediating both behavioral and physiological effects of stress. Clearly then, this research has implications for the effects of stress on the survival of salmonids, particularly during early and critical life history stages. In addition, the generality of the stress response in vertebrates means that these results may also be more widely applicable. In chapter 5 I speculated about the effects of stress on the downstream migration of juveniles. The results of both field studies and the injection studies in that chapter correspond remarkably well. In the field fish tend to delay their migration following exposure to a stressor (Clugston and Schreck, 1992; Snelling and Schreck, 1993). Similarly, in the laboratory I demonstrated that the administration of CRH will delay the downstream movement of several fish, but will also increase the speed of

movement in others. This implies that in the wild, endogenous CRH produced during the response to a stressor not only regulates the production of ACTH and thereby the peripheral responses to stress such as the mobilization of energy, but also may have a role in disrupting the downstream migration. Thus, we are a step closer to identifying why fish behave in ways that may increase their vulnerability to predation. However, it is not immediately apparent why it would be adaptive for a fish to delay their migration. The results of an experiment in Chapter 2 suggest that endogenous CRH may also be beneficial by increasing the ability of the fish to find cover following a stressor. Thus it is reasonable to speculate that the cost of delaying the migration may be outweighed by the benefit of reduced predation at a time when the fish may be physiologically or behaviorally impaired and thus particularly vulnerable. Indeed, many studies have shown that juvenile salmonids are more susceptible to predation following a stressor, although such studies are often done in an environment that does not allow the prey to hide from the predator. It would therefore be interesting to repeat these studies in a more natural environment.

In summary, I have identified several components of the neural system that controls locomotor activity in juvenile salmonids. This is particularly important given that fish represent a very ancient vertebrate model, so that the results obtained using these animals are likely to be applicable to all vertebrates. Furthermore, these results give insight into the conservative nature of many of the systems invovled in the control of locomotor activity from fish to humans.

Interestingly, this work may also represent some middle ground between field studies on wild animals and the many lab studies that have been performed on mammals. In the latter case many of the wildtype behaviors have undoubtably been bred out of the population. That those studies and the work in this thesis tend to corroborate each other suggests that the mechanisms for controlling locomotor activity are highly conserved. I have also made a significant contribution to our understanding of the interaction between CRH and serotonin. This is perhaps the main finding of this thesis as, despite a large effort to understand the control of behavior in mammals there is a surprising paucity of information regarding this interaction. In addition to the control of locomotor activity I have also gathered evidence that these systems are involved in the control of specific behaviors such as habitat choice, sociality, and migration. Again, these results have more general applicability to mammalian vertebrates and further research using this animal as a model may aid our understanding of how such behaviors are controlled in other vertebrates.

Finally, our results have provided clues that will be valuable for directing future research into the effects of stress on the behavior of these fish.

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