2012-04-24

Keeping the Balance: The Role of the Serotonin Subtype 1A Receptor in Regulating Cortisol Secretion in the Gulf Toadfish (Opsanus beta)

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By

Lea R. Medeiros

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

May 2012

UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
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Keeping the Balance: The Role of the Serotonin Subtype 1A Receptor in Regulating Cortisol Secretion in the Gulf Toadfish (*Opsanus beta*) (May 2012)

Abstract of a dissertation at the University of Miami.

Dissertation supervised by Professor M. Danielle McDonald.
No. of pages in text. (131)

It is well established that serotonin (5-HT; 5-hydroxytryptamine) plays a role in regulating the mammalian hypothalamic-pituitary-adrenal (HPA) axis via the 5-HT receptor subtype 1A (5-HT_{1A}). To date, there has not been a comprehensive investigation of the molecular, pharmacological and physiological aspects of the 5-HT_{1A} receptor and its role in the activation of the hypothalamic-pituitary-interrenal (HPI) axis in a single species of teleost fish. The Gulf toadfish (*Opsanus beta*) 5-HT_{1A} receptor was cloned and sequenced, showing 67.5% amino acid similarity to the human homologue. The 5-HT_{1A} receptor was distributed throughout the brain, with the midbrain/diencephalon region containing significantly higher transcript levels than any other brain region. Substantial levels of transcript were also found in the pituitary, while very low levels were found in the kidney, which contains the interrenal cells. *Xenopus* oocyte binding experiments demonstrated that the pharmacology of the Gulf toadfish 5-HT_{1A} receptor is similar to the mammalian form. Confirming these molecular and pharmacological findings, intravenous injection of 8-OH-DPAT, a mammalian 5-HT_{1A} receptor agonist, stimulated the HPI axis to cause a 2-fold increase in circulating levels of cortisol. As the pharmacology and functionality of the toadfish 5-HT_{1A} receptor is similar to the mammalian 5-HT_{1A} receptor, it seemed likely that regulation of the receptor would also be subject to the negative feedback loop that exists in mammals

whereby increased circulating levels of cortisol inhibit 5-HT_{1A} receptor activity. To investigate the possibility of such a feedback mechanism in teleosts, plasma cortisol levels in Gulf toadfish (*Opsanus beta*) were manipulated and the role of cortisol and glucocorticoid receptors (GRs) in the control of 5-HT_{1A} was evaluated. Initial investigations revealed that chronic elevation of plasma cortisol mediates changes in brain 5-HT_{1A} receptor mRNA and protein levels via the GR; however, there appears to be a disconnect between brain levels of the receptor and activation of cortisol release from the interrenal tissue. Targeted investigations confirmed that the release of both corticotropin-releasing factor (CRF) and adrenocorticotropic hormone (ACTH) can be stimulated by activation of centrally located 5-HT_{1A} receptors. Additionally, it was discovered that 8-OH-DPAT-stimulated release of CRF and ACTH was attenuated by crowding stress, and that treatment with RU486 returned secretion rates to control levels. However, while it appears that the GR is responsible for mediating the negative feedback of cortisol on 5-HT_{1A} receptors located in the central nervous system, it does not appear to be responsible for mediating attenuation of cortisol secretion from the interrenal cells. *In vitro* experiments using isolated kidney tissue suggest that chronically elevated plasma cortisol attenuates ACTH- and 5-HT-stimulated cortisol secretion from the interrenal cells of toadfish. Furthermore, the 5-HT_{1A} receptor is not a mediator of cortisol release at the level of the interrenal cells, explaining in part the disconnect between brain 5-HT_{1A} levels and HPI axis function. The series of investigations outlined in this dissertation have provided key information about the functional aspects of the 5-HT_{1A} receptor in a single species, providing a comprehensive examination of the role it plays in both activating and attenuating the stress response in the Gulf toadfish (*Opsanus beta*).

These findings, combined with the fact that cortisol is the predominant corticosteroid in both humans and fish, provide support for considering the Gulf toadfish as a model for the human 5-HT_{1A} receptor.

"Confidence doesn't come out of nowhere. It's a result of something... hours and days and weeks and years of constant work and dedication."

-Roger Staubach

To my mother and father who have always supported my endeavors;
over the years your love and support has instilled in me a confidence that has allowed me to complete this dissertation and know that I can do anything I set my mind to. Thank you

"I learned patience, perseverance, and dedication. Now I really know myself, and I know my voice. It's a voice of pain and victory."

-Anthony Hamilton

To my advisor; I hope this means I am not a loser anymore

Acknowledgements

To my committee: Dr. Danielle McDonald, Dr. Andrew H. Bass, Dr. Lynne A. Fieber, Dr. Martin Grosell, and Dr. Marjorie F. Oleksiak

Thank you for your continued support throughout the process, even when plans changed. Your faith in my progress was truly appreciated and helped me to believe in myself.

Sincere thanks to Mr. Ray Hurley for providing toadfish, without which this dissertation would not have been possible. And I would be nowhere without my friends. Thank you for listening to me, encouraging me when I was down, and getting me drunk when necessary. And to Kirk: thank you for being there for me, no matter where you were.

Funding sources:

NSF Grant IOS-0920547 (to M. Danielle McDonald), The Rowlands fellowship, and The Knight Fellowship

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C	cerebellum
CP	central posterior thalamic nucleus
Df	diffuse nucleus of the hypothalamus
Dl	lateral zone of the dorsal telencephalic area
Dm	medial zone of the dorsal telencephalic area
Dp	posterior zone of the dorsal telencephalic area
DP	dorsal posterior thalamic nucleus
H	hypothalamus
Hc	caudal zone of periventricular hypothalamus
Hind	hindbrain
Hv	ventral periventricular hypothalamus
IL	inferior lobe of the hypothalamus
M	midbrain
OB	olfactory bulb
PGZ	periventricular gray zone of the optic tectum
POA	preoptic area
Pit	pituitary
PM	magnocellular preoptic nucleus
PPp	parvocellular preoptic nucleus, posterior part
PPv	periventricular pretectal nucleus, ventral part
PTN	posterior tuberal nucleus
Sc	suprachiasmatic nucleus
SV	saccus vasculosus
T	telencephalon
Teg	tegmentum
TeO	tectum opticum
TL	torus longitudinalis
TPp	periventricular nucleus of posterior tuberculum
TS	torus semicircularis

CHAPTER 1
THE STRESS RESPONSE AND THE SEROTONIN SUBTYPE 1A RECEPTOR: A LITERATURE REVIEW

Early stress-research pioneer Hans Selye defined two types of stress: "eustress", which occurs every day and does not affect the day-to-day functions of an organism, and "distress", whereby the response may lead to a pathological state that is life-threatening and the organism must adapt or perish (Selye, 1936). Chrousos and Gold (1992) defined stress as the condition in which homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, termed stressors. Stressors work in two ways: they produce effects that threaten or disturb the homeostatic equilibrium and they also elicit a coordinated set of behavioral and physiological responses that are thought to compensate for the imbalance or help the animal to adapt and overcome the threat. However, chronic stress may prevent the animal from fully adapting and lead to dysfunctionality; usually resulting in the retardation of growth, reproductive failure, and reduced immunity to pathogens. Stressors can be specific or general, and while each stressor elicits a characteristic reaction, many different stressors can activate the same pathway (Chrousos and Gold, 1992), achieving the same integrated stress response from a physiological point of view.

Early studies show that the main neuroendocrine control mechanisms of the integrated stress response of fish are comparable to those of mammals and other terrestrial animals, suggesting that these mechanisms conform to a general vertebrate pattern (Donaldson, 1981; Mazeaud, 1981). In teleosts, the stress response involves two systems: the brain-sympathetic-chromaffin cell axis and the hypothalamic-pituitary-

interrenal (HPI) axis (Wendelaar Bonga, 1997). These axes control the stimulation of oxygen (O_2) uptake and transfer, mobilization of energy substrates, reallocation of energy away from growth and reproduction, and suppression of the immune system. Though there are many other factors involved, catecholamines (CAs) and corticosteroids are the primary messengers for the two major systems, with the neuroendocrine system responding in characteristic patterns for each stressor (Mason, 1968). There are substantial differences in etiology between acute and chronic stress that work to affect homeostasis differently, but in general there are three levels involved in the teleost integrated stress response. The primary response involves activation of the brain centers, resulting in the release of CAs and corticosteroids into the circulation. The secondary response is the many different immediate actions and effects of these hormones at the blood and tissue level, as mentioned above. The tertiary response includes the inhibition of growth, reproduction, and the immune response as well as a reduced capacity to tolerate subsequent or additional stressors (Ursin, 1993).

Many of the effects and actions of the stress response are mediated by the HPI axis. This axis is comprised of the hypothalamus, pituitary gland and the interrenal cells (the teleost homologue of the adrenal gland) (Wendelaar Bonga, 1997). In teleosts, the HPI axis can be activated by the hippocampal primordium (analogous to the hippocampus found in mammals) and the amygdala of the diencephalon, which both contain nuclei that pass on stimuli to the hypothalamus, where the message is translated and the appropriate neurochemical response performed (Nieuwenhuys et al., 1998; Flik et al., 2006). Within the diencephalon of teleosts there is a specialized region, termed the preoptic area (POA), where the neuropeptide-secreting neurons of the hypothalamus

reside. This region is analogous to the paraventricular and supraoptic nuclei located in the anterior hypothalamus of tetrapods. Contrary to mammals, teleost fish do not have a hypophysial portal system linking the capillary bed within the hypothalamus to that of the anterior pituitary; instead, the corticotropin-releasing factor (CRF)-containing axons emanating from the POA terminate at the anterior pituitary, releasing CRF and stimulating the secretion of adrenocorticotropic hormone (ACTH) (Peter and Fryer, 1983). ACTH is released by the anterior pituitary into general circulation where it elicits a response via specific receptors present in various organs and tissues. This hormone acts on the interrenal cells, located near the posterior cardinal vein and its branches in the head kidney, to cause the release of corticosteroids in response to stress. While many other hormones, such as atrial natiuretic factor (Arnold-Reed and Balment, 1994), angiotensin (Henderson et al., 1976), growth hormone (Ball and Hawkins, 1976), thyroxin (Young and Lin, 1988), and arginine vasotocin are involved in the axis, CRF and ACTH are the most important secretagogues in terms of corticosteroid secretion.

Cortisol, the primary corticosteroid secreted from the interrenal cells, has a broad activity spectrum, in part due to teleosts lacking aldosterone, the hormone responsible for hydromineral balance in mammals (Agarwal and Mirshahi, 1999). Cortisol provides the same function as aldosterone, serving the purpose of a mineralocorticoid to regulate both hydromineral balance and energy metabolism (Chester Jones, 1980; McCormick, 1995). Although there is no comprehensive model integrating these functions, it is now accepted that cortisol initially plays an adaptive function during stress, but that chronic elevations of cortisol contribute to the deleterious effect of chronic stress (Barton and Iwama, 1991). As with the catecholamines, cortisol has both stimulatory and inhibitory effects on the

physiological functions of fish (Barton, 2002; Wendelaar Bonga, 1997). When released, cortisol initially induces changes in gill chloride cell morphology (Laurent and Perry, 1989), alters intermediary metabolism (e.g. inhibiting protein synthesis) (van der Boon et al., 1991), reduces gut motility and increases liver glycogen concentration (Mommsen, 1999; Wendelaar Bonga, 1997). However, prolonged elevation of glucocorticoids in fish may result in suppression of reproduction, decreased body weight and immunosuppression (Norris, 2000; Wendelaar Bonga, 1997; Weyts et al., 1999). Prolonged administration of glucocorticoids also results in deficits in learning and memory (Lupien and McEwen, 1997) as well as atrophy of neurons in the hippocampi of a number of animal models (Young, 2004). Increased levels of cortisol in the circulation affect carbohydrate, protein and lipid metabolism in an effort to reallocate energy to compensate for the energy demands of the stress. This reduces the performance capacity of the fish during chronic stress as well as the recovery phase following stress (Schreck, 1981; Schreck, 1990). During acute stress, plasma cortisol levels typically rise within a few minutes and return to basal levels in one or more hours; however, chronic stress prevents the return of cortisol to basal levels.

The absence of clearly elevated cortisol levels does not denote a corresponding absence of stressors – the cortisol response can be partially impaired by chronic stress, due to negative feedback (Thomas, 1990) via glucocorticoid (GRs) and mineralocorticoid receptors (MRs). In teleosts, many long-term adaptive changes in response to cortisol are mediated by cytosolic GRs located in target tissues (McDonald et al., 2004; Rodela et al., 2009; Sloman et al., 2001). Furthermore, both GRs and MRs are located throughout the HPI axis (Stolte et al., 2008; Greenwood et al., 2003; Vazzana et al., 2010; Arterbery et

al., 2010) and have been shown to mediate canonical feedback whereby high cortisol levels inhibit the secretion of cortisol (Dallman et al., 2004; Fryer and Lederis, 1986). Immunohistochemistry studies on trout (Tujague et al., 1998) have revealed GR-immunoreactive cells in the in the POA and the pituitary (Fuxe et al., 1985), supporting the idea of a long-loop negative feedback mechanism in which CRF-producing neurons and ACTH secretion are inhibited by corticosteroids (Fryer et al., 1984; Rotllant et al., 2000b). However, there is no study that specifically examines the role of the GR in mediating cortisol-induced decreases in CRF and ACTH secretion. There is also evidence that corticosteroids have a self-suppression effect directly at the level of the interrenal cells (Bradford et al., 1992; Rotllant et al., 2000b; Vijayan and Leatherland, 1990; Young, 1986). Bradford et al. (1992) and Young (1986) have utilized *in vitro* methods to demonstrate the existence of ultra-short-loop feedback mechanism in salmonids, in which the steroidogenic effects of ACTH on isolated head kidney cells, although dramatic, appear to be short-lived as cortisol accumulated in the incubation media and inhibited further secretion. While Bradford et al. (1992) identified that it was the accumulation of cortisol in the bath which inhibited continuous secretion in coho salmon, and not an exhaustion of precursor or degradation of the cells, the group did not determine what was mediating this inhibition.

In mammals, it is well established that the monoamine neurotransmitter serotonin (5-HT; 5-hydroxytryptamine) stimulates the secretion of cortisol from the adrenal gland both directly and also by initiating the release secretagogues, such as CRF and ACTH (Chaouloff, 1993; Dinan, 1996). Additionally, it has been shown that brain 5-HT synthesis and turnover is greatly impacted by stressful events (Bliss et al., 1972),

identifying it as a target for the negative feedback of cortisol. Thus, brain 5-HT could play an integral role in a complex neuroendocrine loop serving to maintain homeostasis and promote acclimation during physiological and/or environmental challenges. Due to conserved patterns of brain organization among vertebrates (Parent, 1981; Parent et al., 1984; Nieuwenhuys et al., 1998) it is likely that the serotonergic system was also conserved (Winberg and Nilsson, 1993). As aforementioned, each stressor elicits a characteristic stress pattern, and this holds true for the role 5-HT plays in mediating the stress response. Social stressors, such as subordination, elevate brain 5-HT activity as indicated by brain 5-hydroxyindoleacetic acid (5-HIAA; the major 5-HT metabolite) concentrations and 5-HIAA:5-HT ratios (Winberg and Nilsson, 1993). Socially subordinate fish display elevated plasma cortisol levels (Ejike and Schreck, 1980) and increased interrenal cell sizes, suggesting a chronic activation of the HPI axis (Noakes and Leatherland, 1977). This supports the theory that the teleost serotonergic system plays a similar role in the stress response as it does in mammals. As such, due to the fact that the stimulatory role of 5-HT on the hypothalamic-pituitary-adrenal (HPA) axis has been attributed to 5-HT receptor subtypes 1A (5-HT_{1A}) and 2 (5-HT_2) (Dinan, 1996) in the central nervous system as well as the 5-HT_4 receptor in the adrenal gland (Hegde and Eglen, 1996), it is likely that the same receptors play a role in the stimulation of the HPI axis in teleosts.

Serotonin receptor subtype 1A is one of the most abundant subtypes present in the mammalian brain (Albert et al., 1990). The receptor employs a G_i/G_O transduction system that decreases adenylate cyclase formation and/or increases K^+ conductance (Barnes and Sharp, 1999), and closing of Ca^{2+} channels, or other coupling mechanisms,

may also occur (Fargin et al., 1991). These mechanisms are postulated to inhibit firing of the postsynaptic cell, and it has been observed that 5-HT does exert a predominantly inhibitory effect on neuron firing rate in many areas of the brain (Araneda and Andrade, 1991; Clark et al., 1987; Davies et al., 1987; Kow et al., 1992; Newberry and Priestley, 1988), and most attribute these inhibitory effects to the activation of 5-HT_{1A} receptors. Even so, there are two types of 5-HT_{1A} receptors: somatodendritic autoreceptors and postsynaptic receptors. Activation of the somatodendritic 5-HT_{1A} autoreceptors, located in the raphe nuclei, reduces synthesis of 5-HT, decreases firing of 5-HT neurons, and decreases release of 5-HT from synaptic terminals (Sprouse and Aghajanian, 1987; Verge et al., 1985). Stimulation of 5-HT_{1A} autoreceptors has an anxiolytic, or anti-anxiety effect. Postsynaptic 5-HT_{1A} receptors, on the other hand, are located in the target areas of the raphe nuclei (*i.e.*, the telencephalon, hypothalamus, and pituitary) and have an anxiogenic, or anxiety-promoting, effect when activated (File et al., 1996). While the two types of 5-HT_{1A} receptors are located in different regions of the brain, they are still far from independent. The 5-HT_{1A} somatodendritic receptors are in partial control of the postsynaptic receptors; these autoreceptors regulate the release of 5-HT by the presynaptic neuron, ultimately affecting the stimulation of 5-HT_{1A} receptors on the postsynaptic cell.

While molecular evidence for the presence of 5-HT_{1A} receptors in fish (Airhart et al., 2007; Wang and Tsai, 2006; Yamaguchi and Brenner, 1997) exists and studies have provided pharmacological evidence for a role for 5-HT_{1A} in teleosts (Clotfelter et al., 2007; Smith and Combs, 2008; Winberg et al., 1997) no single study has reported molecular, pharmacological and physiological characterization of the teleost 5-HT_{1A}

receptor. Agonists of mammalian 5-HT_{1A} receptors, such as 8-hydroxy- 2-(di-n-propylamino) tetraline (8-OH-DPAT), have been found to elevate plasma corticosteroid levels in mammals, a result that has also been observed in teleosts (Bovetto et al., 1996; Fuller, 1990; Van de Kar et al., 1989; Winberg et al., 1997), further corroborating the mounting evidence that the teleost 5-HT_{1A} receptor is much the same as the mammalian (Winberg and Nilsson, 1993; Zifa and Fillion, 1992). This conclusion is further supported by the fact that a previous study clearly showed that 5-HT_{1A}-like receptors are present in the brain of salmonid fish and exhibit pharmacological properties similar to the 5-HT_{1A} receptor found in mammals (Winberg and Nilsson, 1996). Later studies by the same group showed a trend for 8-OH-DPAT to increase plasma ACTH levels, which was followed by a significant increase in plasma cortisol (Höglund et al., 2002). However, at this time, it is not obvious at which level of the HPI axis 8-OH-DPAT is exerting its effect as no study has addressed the role of 5-HT_{1A} receptors in mediating CRF secretion in teleosts. Thus, it is clear that 5-HT stimulates glucocorticoid secretion via the 5-HT_{1A} receptor in both mammals and teleost fish.

In mammals, there is also evidence for a homeostatic role for glucocorticoids in maintaining 5-HT_{1A} receptor level and function. There is also evidence for the presence of a negative feedback loop; adrenalectomy, which removes background levels of glucocorticoids, increases [^{3}H]8-OH-DPAT binding in the CA2 region of the hippocampus (Mendelson and McEwen, 1992), and highly elevated circulating levels of glucocorticoids inhibit 5-HT_{1A} receptor activity (Zhong and Ciaranello, 1995). Specifically, in mammals, a decreased expression of 5-HT_{1A} mRNA in response to elevated glucocorticoids is correlated with a decrease in binding (Laaris et al., 1995) and

function (Porter et al., 1998), which is thought to be mediated via the GR and not the MR (de Kloet et al., 2009; Meijer and de Kloet, 1994). Furthermore, elevated glucocorticoids have been shown to downregulate 5-HT_{1A} function independent of transcript level or protein activity (Van de Kar et al., 1998). As stimulation of the 5-HT_{1A} receptor ultimately results in the secretion of cortisol, it is plausible to surmise that a decrease in 5-HT_{1A} receptor activity would result in a decrease in CRF, ACTH, and cortisol secretion. Indeed, there is evidence for corticosteroids directly inhibiting the release of CRF and ACTH in both mammals and fish (Canny et al., 1989; Delbende et al., 1992; Fryer and Peter, 1977; Rotllant et al., 2001; Rotllant et al., 2000b), though the decreases have not been studied in reference to the 5-HT_{1A} receptor.

Since mounting evidence suggests that the serotonergic system appears to be highly conserved between mammals and teleosts, it is likely that chronic elevations in glucocorticoids may also suppress the 5-HT_{1A} receptor in teleosts as it does in mammals (Chalmers et al., 1993; Meijer and de Kloet, 1994), and in doing so, inhibit the 5-HT_{1A}-mediated secretion of CRF and ACTH. As the GR has been identified as a likely mediator of the effects of cortisol on the 5-HT_{1A} receptor in mammals, and considering the high conservation of the 5-HT system in vertebrates, it is likely that the GR is also mediating such a feedback loop in teleosts; however, this theory remains untested. Using a whole-animal approach, the series of studies outlined in this dissertation attempt to localize and characterize the 5-HT_{1A} receptor on a molecular, pharmacological and physiological level in a single species of teleost fish, while simultaneously determining if cortisol appears to be mediating a decrease in the receptor's functionality on all levels of the HPI axis.

All of the investigations in this dissertation were conducted on the Gulf toadfish, *Opsanus beta*, and all protocols were approved by the University of Miami IACUC. The Gulf toadfish is extremely robust, recovers quickly after surgery, and is relatively easy to maintain; making it an excellent experimental organism that is readily available in Biscayne Bay, FL. The findings of the present study, combined with the fact that cortisol is the predominant corticosteroid in both humans and fish, may also reveal the Gulf toadfish as an excellent model for the human 5-HT_{1A} receptor.

CHAPTER 2
THE SEROTONIN SUBTYPE 1A RECEPTOR REGULATES CORTISOL SECRETION IN THE GULF TOADFISH, *OPSANUS BETA*[1]

Background & significance

The monoamine neurotransmitter, serotonin (5-HT; 5-hydroxytryptamine), affects many systems in the body, mediating the physiological processes that regulate anger, mood, sleep, appetite, and even learning (Barnes and Sharp, 1999; Fuller, 1990; Uphouse, 1997). Serotonergic activation of the hypothalamic-pituitary-adrenal (HPA) axis, an important component of the mammalian stress response, results in an increase in levels of corticotropin-releasing hormone (CRH) from the hypothalamus and stimulates the secretion of adrenocorticotropic-releasing hormone (ACTH) from the pituitary, which then activates the secretion of glucocorticoids, such as cortisol, from the adrenal gland (Calogero et al., 1990). In addition to the indirect stimulation of gluococorticoid secretion via CRH and ACTH (Calogero et al., 1990), 5-HT also activates the release of cortisol directly by acting on the adrenal gland; however, this increase is thought to be independent of the HPA axis with 5-HT acting as a local paracrine factor (Alper, 1990). Reciprocally, it has been shown that brain 5-HT synthesis and turnover is greatly impacted by stressful events (Bliss et al., 1972), which, in turn, will feedback on the HPA axis. Thus, brain 5-HT plays an integral role in a complex neuroendocrine loop serving to maintain homeostasis and promote acclimation during physiological and/or environmental challenges. It was not until the last few decades that the connection between the HPA axis, 5-HT and the 5-HT receptor subtype 1A (5-HT_{1A}) was made (Fuller, 1992; Lorens and Van de Kar, 1987).

[1] This chapter has been published previously in General and Comparative Endocrinology. Please see Medeiros et al., 2010 for the full citation.

Within the CNS, 5-HT binds to several different types of 5-HT receptors (HTRs), located both pre- and postsynaptically. To date, seven families of HTRs have been identified (5-HT_1-5-HT_7), with a total of 14 subtypes having been characterized (Hoyer et al., 2002). All families are G protein-coupled receptors, with the exception of 5-HT_3, which is a ligand-gated ion channel. The 5-HT_1 family inhibits the formation of cyclic AMP (cAMP), whereas 5-$HT_{4,6,7}$ families stimulate the production of cAMP. The 5-HT_2 family communicates via the second messenger phospholipase C, while the mechanism whereby the 5-HT_5 family acts remains a little obscure (see Hoyer et al., 2002). While all of these receptors have a specific and important purpose, the 5-HT_{1A} receptor is one of the most abundant subtypes of 5-HT receptors in the mammalian brain (Albert et al., 1990) and, being the first HTR cloned (Kobilka et al., 1987), has been the most studied. The G-protein-coupled receptor employs a G_i/G_0 transduction system that primarily decreases adenylate cyclase formation and/or increases K^+ conductance (Barnes and Sharp, 1999). These mechanisms are postulated to inhibit firing of the postsynaptic cell, and it has been observed that 5-HT, mediated by the 5-HT_{1A} receptor, exerts a predominantly inhibitory effect on neuron firing rate in many areas of the brain (Araneda and Andrade, 1991; Clark et al., 1987; Kow et al., 1992). There are two populations of 5-HT_{1A} receptors: somatodendritic autoreceptors located mainly in the rostral raphe nucleus region of the medulla and postsynaptic receptors, located in the projection areas of the raphe nuclei, such as the telencephalon and hypothalamic/pituitary region. The somatodendritic 5-HT_{1A} autoreceptors regulate the release of 5-HT by the presynaptic neuron, ultimately affecting the stimulation of 5-HT_{1A} receptors on the postsynaptic cell and thus the activation of the 5-HT_{1A} neuron projection areas in the brain (Albert and

Lemonde, 2004; Lanfumey and Hamon, 2000). Agonists of mammalian 5-HT_{1A} receptors, such as 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT), have been found to elevate plasma corticosteroid levels in mammals (Dinan, 1996). Depending on the location of receptor, this endpoint can be achieved in several different ways, *i.e.*, when applied directly to the rat hypothalamus, 8-OH-DPAT stimulates the release of CRH, though when applied directly to the pituitary, ACTH is released (Calogero et al., 1990). There is also evidence for the presence of a negative feedback loop whereby increased circulating levels of cortisol inhibit 5-HT_{1A} receptor activity (Zhong and Ciaranello, 1995) in addition to directly inhibiting the release of ACTH and CRH (Canny et al., 1989; Delbende et al., 1992).

Work done on teleost fish investigating the interaction between 5-HT and the teleost homologue of the HPA axis, the hypothalamic-pituitary-interrenal (HPI) axis, has mostly focused on how these systems work together during social interactions (Clotfelter et al., 2007; Larson et al., 2003a; Winberg and Nilsson, 1993). Social stressors, such as subordination, elevate brain 5-HT activity as indicated by brain 5-hydroxyindoleacetic acid (5-HIAA; the major 5-HT metabolite) concentrations and 5-HIAA:5-HT ratios (Winberg and Nilsson, 1993). At the same time, socially subordinate fish display elevated plasma cortisol levels (Ejike and Schreck, 1980) and increased interrenal cell size, suggesting a chronic activation of the HPI axis (Noakes and Leatherland, 1977). Work done on the brain of Arctic Charr (*Salvelinus alpinus*) suggested that, like in mammals, there are a multitude of 5-HT receptors in teleost fish to carry out the actions of 5-HT (Winberg and Nilsson, 1996). Furthermore, similar to its action in mammals, the 5-HT_{1A} receptor agonist, 8-OH-DPAT, has been shown to stimulate the release of

cortisol in the rainbow trout (Oncorhynchus mykiss, Winberg et al., 1997), with a binding affinity (K_i) similar to what is measured in mammals (Zifa and Fillion, 1992).

While molecular evidence for the presence of 5-HT_{1A} receptors in fish (Airhart et al., 2007; Wang and Tsai, 2006; Yamaguchi and Brenner, 1997) exist and studies have provided pharmacological evidence for a role for 5-HT_{1A} in teleosts (Clotfelter et al., 2007; Smith and Combs, 2008; Winberg et al., 1997) no single study has reported molecular, pharmacological and physiological characterization of the teleost 5-HT_{1A} receptor. Thus, previous to this study there has not been an extensive investigation that has determined conclusively that the 5-HT_{1A} receptor in fish is pharmacologically similar to that found in mammals and that it plays a role in the activation of the HPI axis. Furthermore, the distribution of 5-HT_{1A} transcript within the HPI axis has not been investigated in teleost fish, although it can be hypothesized based on mammalian studies that most of the 5-HT_{1A} transcript would be found in the pituitary gland (Chalmers and Watson, 1991; Drevets et al., 2007; Kumar and Mann, 2007; Lopez et al., 1998). Preliminary pharmacological evidence on the Gulf toadfish, *Opsanus beta*, a benthic marine teleost fish suggested that a 5-HT_{1A}-like receptor may not be involved in the regulation of the HPI axis, as fish injected with 8-OH-DPAT failed to show an increase in plasma cortisol levels that exceeded the increase measured in saline-injected controls (McDonald and Walsh, 2004). However, it was speculated in that study that the high circulating cortisol levels experienced typically by cannulated toadfish (200-400 x 10^{-9} $g \cdot ml^{-1}$) may have reduced the sensitivity of the 5-HT_{1A} receptor, making it difficult to further stimulate the HPI axis in these fish. We hypothesize now that the 5-HT_{1A} receptor is indeed present in toadfish, but perhaps a higher dose of 8-OH-DPAT is

necessary to overcome this potential desensitization. Thus, the objectives of this study were to examine the existence of the 5-HT_{1A} receptor in the Gulf toadfish, and specifically determine its distribution within the HPI axis, and at the same time provide functional evidence linking the toadfish 5-HT_{1A} receptor to the activation of the HPI axis. To do this, we first determined the full length nucleotide sequence of the toadfish 5-HT_{1A} receptor; we then evaluated the distribution of 5-HT_{1A} receptor transcript levels within different tissues, including those of the HPI axis. Lastly, we set out to provide pharmacological and functional evidence linking the 5-HT_{1A} receptor to HPI axis regulation.

Materials and Methods

Experimental animals

Gulf toadfish (*Opsanus beta*) were captured by roller trawl used by commercial shrimpers in Biscayne Bay, Florida in the summer of 2007, after which they were then immediately transferred to the laboratory where they were held for up to one month. Fish were treated with a dose of malachite green (final concentration 0.05 mg l^{-1}) in formalin (15 mg l^{-1}) (AquaVet) on the day of transfer to the laboratory in order to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993). The fish were kept in 50-liter glass aquaria with flowing, aerated seawater at a temperature of 24-29ºC and were fed weekly with squid.

Experimental protocols

RNA extractions. Tissues were excised from toadfish that had been held for one week in uncrowded conditions in outdoor 6000 L tanks seeded with the seagrass, *Thalassia testudinum* which emulates the natural environment of Gulf toadfish (Serafy et al., 1997). Toadfish were over-anesthetized with MS222 (3 $g \cdot L^{-1}$) and tissues were collected terminally, frozen immediately in liquid N_2 and stored at –80°C. Total RNA was isolated from tissues following the protocol provided with the Trizol reagent (Invitrogen). Total RNA was subsequently treated with DNAse I to remove potential residual genomic DNA according to the protocol provided with the TurboDNA-free kit (Ambion).

PCR and 5' and 3' rapid amplification of cDNA ends (RACE). Toadfish poly(A) RNA was extracted from the total RNA using the PolyATract mRNA Isolation System III (Promega) for use in RACE reactions. cDNA was synthesized with Oligo(dT) primers from 1 μg of DNase I-treated total RNA according to the protocol provided with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). An initial fragment of 779 bp was cloned from toadfish brain cDNA using degenerate primers (Table 1.1) designed using alignments of other teleosts. Reactions were performed using GoTaq DNA polymerase (Promega) and the following cycling conditions: 94°C for 30 s, a temperature gradient of 50-70°C for 1 min, and 72°C for 1 min for 40 cycles. To obtain the 5' and 3' ends, 1μg of poly(A) RNA was amplified into RACE-ready cDNA using the BD SMART RACE kit (Clontech). Primers for use in RACE were designed from the previously acquired toadfish sequence (Table 2.1). Touchdown PCR was performed using the following cycling parameters: five cycles of 94°C for 30 s and 72°C for 4 min,

followed by five and then 25 additional cycles as outlined above with annealing temperatures of 70°C and 68°C, respectively. A second round of amplification was performed with diluted aliquots (1:100) of the initial PCR reactions using nested primers (Table 2.1) by repeating the cycle conditions above, except with only a 2.5 min elongation period. Products were gel-purified, cloned using the TOPO TA vector (Invitrogen) and sequenced (Geneway Sequencing; Hayward, CA).

Table 2.1. Primers used for qPCR and cloning of the Gulf toadfish 5-HT_{1A} receptor

Primer	Sequence (5'→3')	Product Size (bp)
5-HT_{1A}-F*	TAYCARGTSYTNAAYAAGTG	857
5-HT_{1A}-R*	TANCCNARCCARTTRATNAC	
5-HT_{1A}-5' RACE	GTTTACGCACGGAGAGTTGGCTTTAGG	786
5-HT_{1A}-n5' RACE	GATGAACAGGTCGCAGAGCTCCTG	306
5-HT_{1A}-3' RACE	CAGGAGCTCTGCGACCTGTTCATC	1100
5-HT_{1A}-n3' RACE	CTCTTGATTAGCGTGACTTGGTTAATTGGTTTC	915
qPCR 5-HT_{1A} F	ACGAAACCATGAACGAAAGG	119
qPCR 5-HT_{1A} R	AAGGGAAGCCAGCAGAAGAT	
18S-F	GCTCGTAGTTGGATCTCGG	166
18S-R	GGCCTGCTTTGAACACTC	
ORF 5-HT_{1A}-F	ATGGATTTTGTGACAAGCAGCAACG	1253
ORF 5-HT_{1A}-R	TTAAGCTCTGTGGAATTTGC	

***Primer sequences used for initial cloning of toadfish 5-HT_{1A} fragment, designed from partially conserved regions of other teleost 5-HT_{1A} aligned sequences**
Abbreviations: forward primer (F); reverse primer (R); nested primer (n); open reading frame (ORF)

Quantitative PCR (qPCR). A tenfold dilution of cDNA was made up using molecular biology grade water (Sigma-Aldrich). Housekeeping gene primer sequences for 18S were obtained from Grosell et al. (Grosell et al., 2009). qPCR was performed using a Mx4000 Multiple Quantitative PCR system (Stratagene) with SYBR Green qPCR Master Mix (Applied Biosystems) as the reporter dye. Cycling parameters were as follows: 95°C for 10 min, followed by 40-50 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. At least 6 separate biological replicates, representing cDNA isolated from at least 6 individual fish, were used for each sample. Fold-changes between tissues and brain regions were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Expression of 18S appeared stable across different brain regions, but varied significantly among the other tissues examined throughout the organism. Therefore, within the brain $5HT_{1A}$ expression was normalized to 18S, while expression in other tissues was normalized to the amount of total RNA used for cDNA synthesis (1 μg). Fold-changes in mRNA expression between tissues were assessed relative to the brain and changes between brain regions were assessed relative to the telencephalon.

Binding and pharmacological specificity for toadfish 5-HT$_{1A}$ receptors expressed in Xenopus *oocytes*. The full-length toadfish 5-HT_{1A} receptor was cloned into the pGH19 vector (a kind gift from Dr. Gerhard Dahl, University of Miami, Miller School of Medicine, Miami, FL, USA) and linearized with *XhoI*. cRNA was synthesized using the mMessage mMachine T7 Transcription Kit (Ambion) and microinjected (1 μg) into collagenase-treated, defolliculated *Xenopus laevis* oocytes as described previously (Lindley et al., 2007). Parallel groups of oocytes were injected with water as a negative

control. The binding experiment was conducted 36 h after injection and involved five parts: *(i)* binding of 1.35 x $10^{-9}\cdot L^{-1}$ [^{3}H]-5-HT by 5-HT_{1A}-injected oocytes compared to water-injected controls; *(ii)* the displacement or competition of 1.35 x $10^{-9}\cdot L^{-1}$ [^{3}H]5-HT binding by 1.35 x $10^{-9}\cdot L^{-1}$ 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT; Sigma-Aldrich), an agonist specific for mammalian 5-HT_{1A} receptors; (*iii*) evaluation of 5-HT binding affinity using [^{3}H]5-HT concentrations varying from 0.0-5.0 x $10^{-9}\cdot L^{-1}$; (*iv*) determination of an EC50 for 8-OH-DPAT using the displacement or competition of 1.00 x $10^{-9}\cdot L^{-1}$ [^{3}H]5-HT binding by 8-OH-DPAT ranging in concentration from 0.0-10.0 x $10^{-9}\cdot L^{-1}$; and (*v*) the displacement or competition of 1.0 x $10^{-9}\cdot L^{-1}$ [^{3}H]5-HT binding by 1.80 x 10^{-11} $mol\cdot L^{-1}$ 8-OH-DPAT, SB269970 (a 5-HT_7 antagonist; Sigma-Aldrich), α-methylserotonin (a 5-HT_2 agonist; Sigma-Aldrich), or 5-nonyloxytryptamine (a 5-HT_{1B} agonist; Tocris). A separate control treatment was evaluated with 5-nonyloxytryptamine due to it only being soluble in 100% EtOH; serial dilutions put the final EtOH concentration at 0.01%. Injected oocytes were pre-incubated in buffer (in mM: 200 mannitol, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, 5 Tris, pH 7.4) for 15 minutes and then transferred to a binding solution consisting of the pre-incubation buffer containing [^{3}H]5-HT (American Radiolabeled Chemicals, Inc.) with or without the appropriate serotonin receptor agonist or antagonist. Oocytes were incubated for 60 min and then removed from the incubation bath and rinsed twice with pre-incubation buffer. Oocytes were placed individually into 20 ml scintillation vials and immediately digested in 10% SDS (1 ml) overnight. After digestion, 10 ml of Ecolume scintillation fluor (MP Biomedical) was added to each vial and radioactivity was measured by liquid scintillation counting (Tri-Carb 2100TR Liquid Scintillation Counter, Packard). Non-specific binding was

subtracted from total binding before making comparisons from all treatments except when comparing the binding of [^{3}H]5-HT in water-injected oocytes to those injected with 5-HT$_{1A}$ cRNA.

In vivo *injection of 8-OH-DPAT to determine potential presence of the 5-HT$_{1A}$ receptor in gulf toadfish.*

Toadfish (80 ±5 g; N=9) were anesthetized with MS222 (1 g·L^{-1}; Argent Chemical Laboratories) and fitted with caudal vein catheters inserted using surgical techniques previously described (McDonald et al., 2000; Wood et al., 1997). Catheterized fish were allowed to recover for at least 40 hours before injection with 8-OH-DPAT (Sigma-Aldrich). Responsiveness of the HPI axis to two different doses of 8-OH-DPAT (3.25 and 16.25 mg·kg^{-1}·mL^{-1} saline, which represented doses calculated to achieve circulating 8-OH-DPAT concentrations that were 10- and 50-times higher than circulating levels of 5-HT, respectively) were tested. These doses were based on the lack of an effect of 8-OH-DPAT injection to stimulate a cortisol response that was significantly different than that elicited by serial blood sampling alone at a dose of 0.325 mg·kg^{-1}·mL^{-1} saline (McDonald and Walsh, 2004). Immediately following the collection of a 200 µL blood sample (t = 0 h), 8-OH-DPAT was administered via the caudal catheter. Subsequent blood samples (200 µL) were taken at t=0.25, 0.5, 1, 2, and 4 h post-8-OH-DPAT injection. After each sample was spun down, red blood cells were resuspended in 200 µL saline (150 mmol·l^{-1} NaCl) and injected back into the fish. Collected samples were centrifuged at 10,000 *g* for 5 minutes and the plasma decanted. Plasma from each sample was flash frozen in liquid N_2, and then stored at -80°C until being used to evaluate

circulating levels of the stress hormone, cortisol, and products of gluconeogenesis such as circulating levels of urea, protein and glucose.

Organic Compound Assays. Plasma glucose concentrations were measured using a commercial Hexokinase kit (Stanbio Laboratory), with standards ranging from 0-25 $mmol \cdot L^{-1}$. Circulating levels of protein were assessed using Bradford Assay (Sigma-Aldrich), with bovine protein standard (Sigma-Aldrich) diluted to a toadfish-appropriate range of 0-0.06 $g \cdot 100mL^{-1}$. Plasma urea levels were measured using the diacetyl monoxime method of Rahmatullah and Boyde (Rahmatullah and Boyde, 1980), with changes to reagent strength appropriate for plasma urea concentrations found in toadfish. Plasma cortisol was quantified using a ^{125}I RIA kit (MP Biomedical), with the standards diluted by half to be appropriate for the plasma protein range of toadfish.

Statistical Analyses. All binding curve analyses of *in vitro* data were determined using GraphPad Prism v5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All other analyses were conducted using SigmaStat and/or SigmaPlot (SPSS, Inc.). Data are given as means ± 1 s.e.m., and N=the number of fish or oocytes. The *in vivo* response to 8-OH DPAT was analyzed with a one-way ANOVA with time as the main factor and multiple comparisons were conducted using the Holm-Sidak method ($p<0.05$). All other comparisons were conducted using either the Student's unpaired two-tailed *t*-test ($p<0.05$) or, if data were not normally distributed even after log transformation, the Mann-Whitney rank sum test ($p<0.05$).

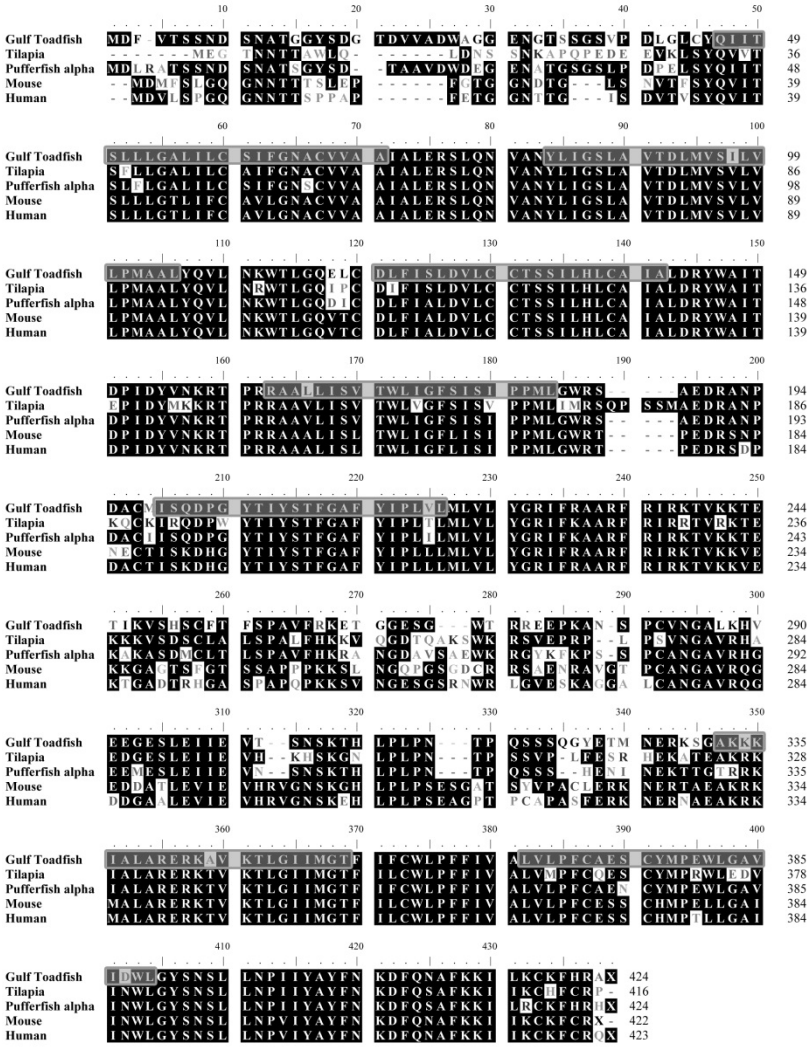

Figure 2.1. CLUSTAL (v1.8) amino acid alignment of translated nucleotide 5-HT$_{1A}$ receptor sequences from human (*homo sapiens*), NM000524; mouse (*Mus musculus*), MMU39391; Fugu (*Takifugu rubripes*), X95936; Tilapia (*Oreochromis mossambicus*), AY219038; and Gulf toadfish (*Opsanus beta*), FJ769221. Outlined amino acids indicate a transmembrane helix, as predicted by HMMTP (v2.0; http://www.enzim.hu/hmmtop/index.html).

Results

The toadfish 5-HT$_{1A}$ receptor transcript (GenBank Accession# FJ769221) is 1,824 nucleotides long, and codes for 423 amino acids. Upon analysis, it was determined that the predicted amino acid sequence shares 67.5% homology to the human 5-HT$_{1A}$

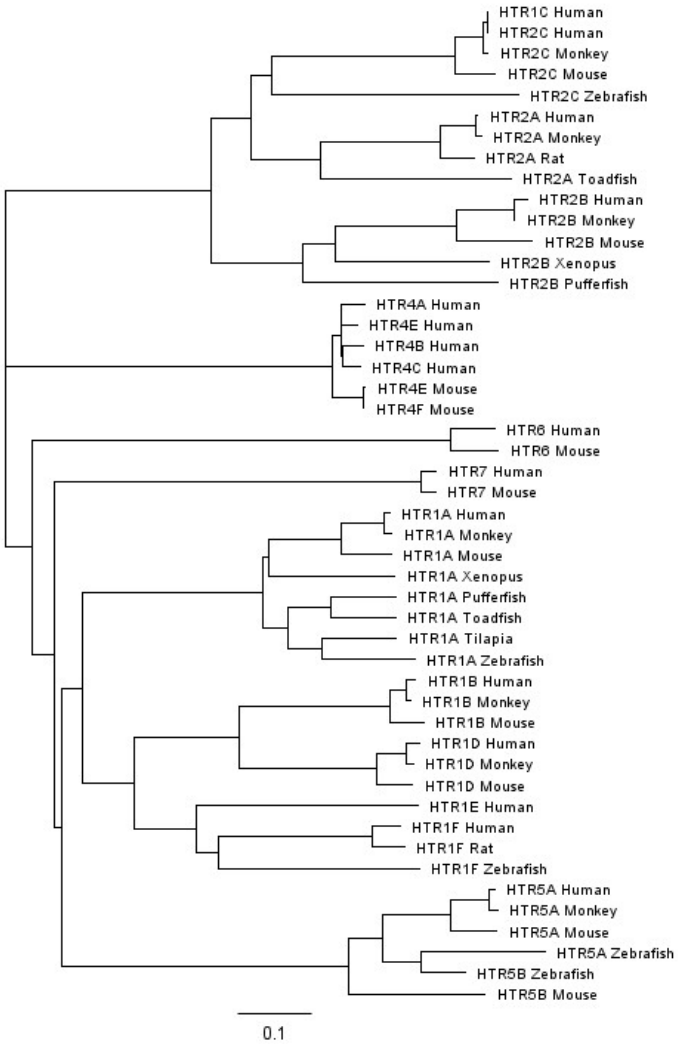

Figure 2.2. Phylogenetic analysis of 48 5-HT receptor sequences. Sequences were translated, aligned, and the tree constructed using Geneious Basic (v4.6.4) and the Neighbor Joining method with bootstrap analysis.

sequence, 67.6% to the mouse, 69.2% to tilapia, and 82.4% to pufferfish (Fig. 2.1). Phylogenetic analysis of all 5-HT receptors resulted in placing the nucleotide and amino acid sequence for toadfish 5-HT_{1A} in the 5-HT_{1A} receptor branch (Fig. 2.2). The toadfish sequence had the highest similarity to the pufferfish, but fell out with the other teleosts in

the same sub-branch of the 5-HT$_{1A}$ group. Of note, there were two sub-branches in the 5-HT$_{1A}$ grouping, with the teleosts and terrestrial vertebrates separated. Furthermore, the terrestrial vertebrates were separated into amphibians and mammals, validating the

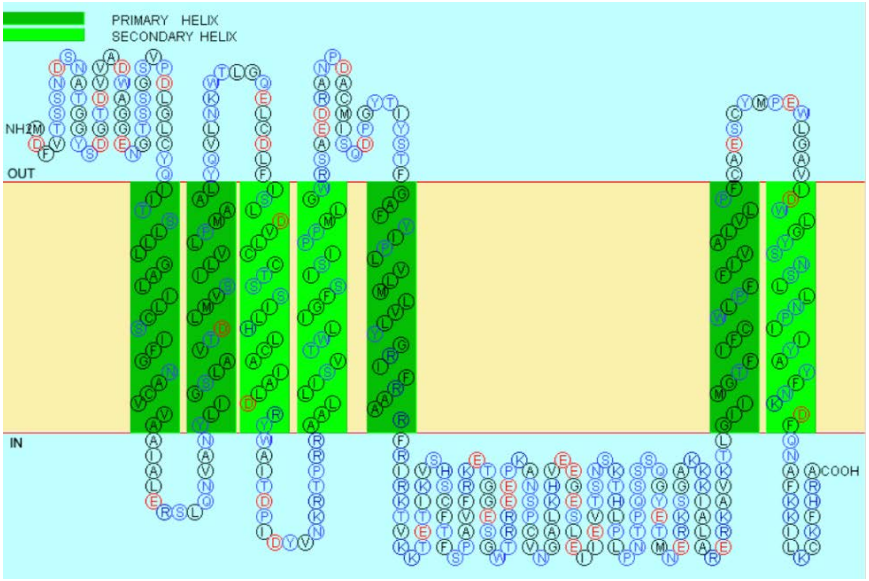

Figure 2.3. Predicted amino acid sequence and resulting membrane structure of Gulf toadfish 5-HT$_{1A}$ receptor predicted by the SOSUI prediction system (http://bp.nuap.nagoya-u.ac.jp/sosui/).

sequence analyses. Predicted protein folding using the SOSUI prediction system (http://bp.nuap.nagoya-u.ac.jp/sosui/) revealed 7 transmembrane regions with the N-terminus on the extracellular side (Fig. 2.3).

The 5-HT$_{1A}$ receptor was found to be expressed in many tissues, though predominantly in the brain and reproductive system, as well as the swim bladder (Fig. 2.4). The brain possessed approximately 2-fold the amount of 5-HT$_{1A}$ receptor mRNA as the swim bladder, which had the second highest expression of the transcript (Fig. 2.4A). The gonad had the third highest level of expression, though only about 20% of the transcript measured in the brain; and had a similar amount of expression compared to

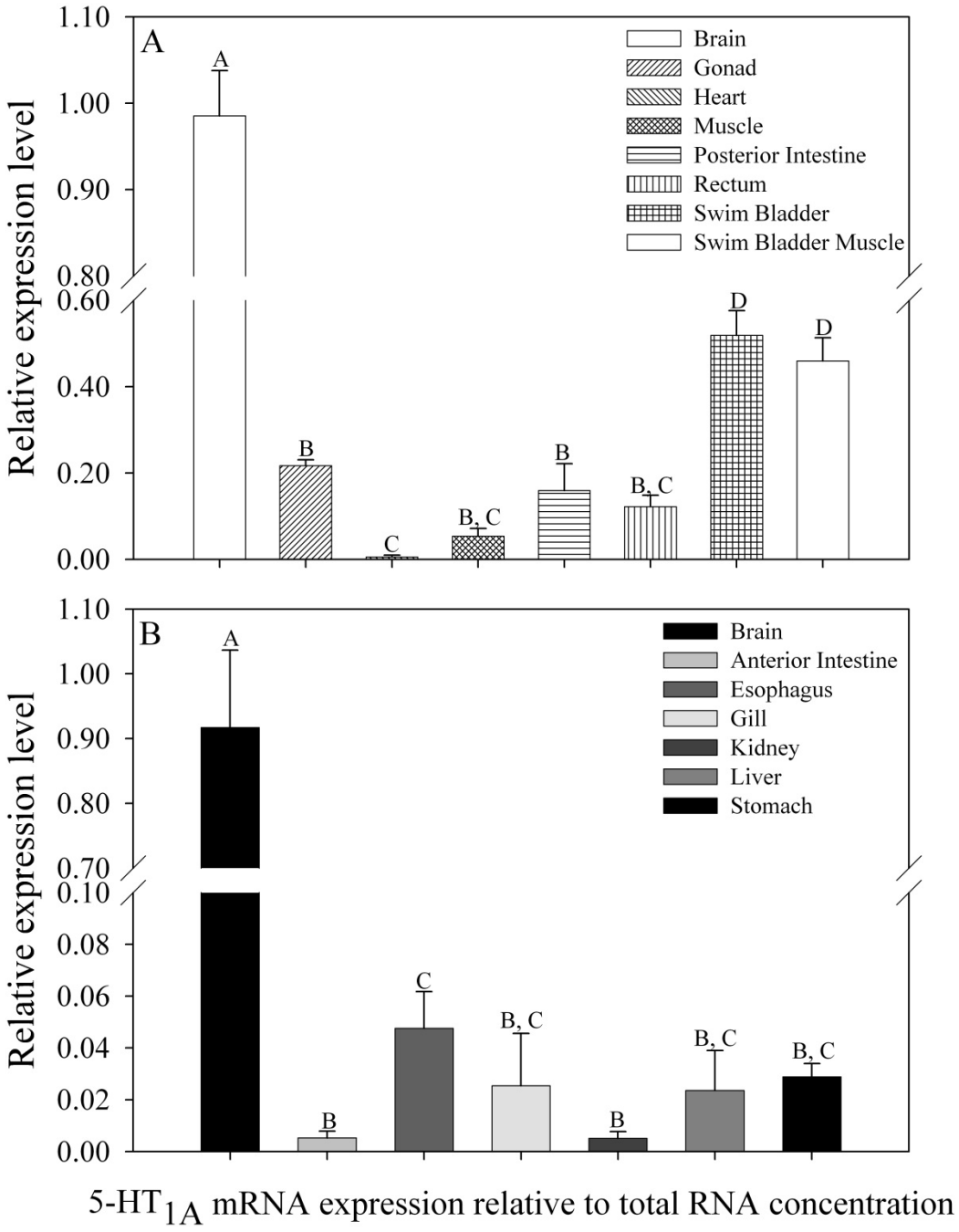

Figure 2.4. Relative quantity of 5-HT_{1A} mRNA expression present in various tissues of the Gulf toadfish, *Opsanus beta*. (A) Transcript levels in gonad, heart, muscle, posterior intestine, rectum, swim bladder, swim bladder muscle relative to the brain from one subset of fish (N = 6) and (B) transcript levels from anterior intestine, esophagus, gill, kidney, liver and stomach relative to brain from a second set of fish. Values are means ± s.e.m. A different letter depicts significant difference ($p < 0.05$).

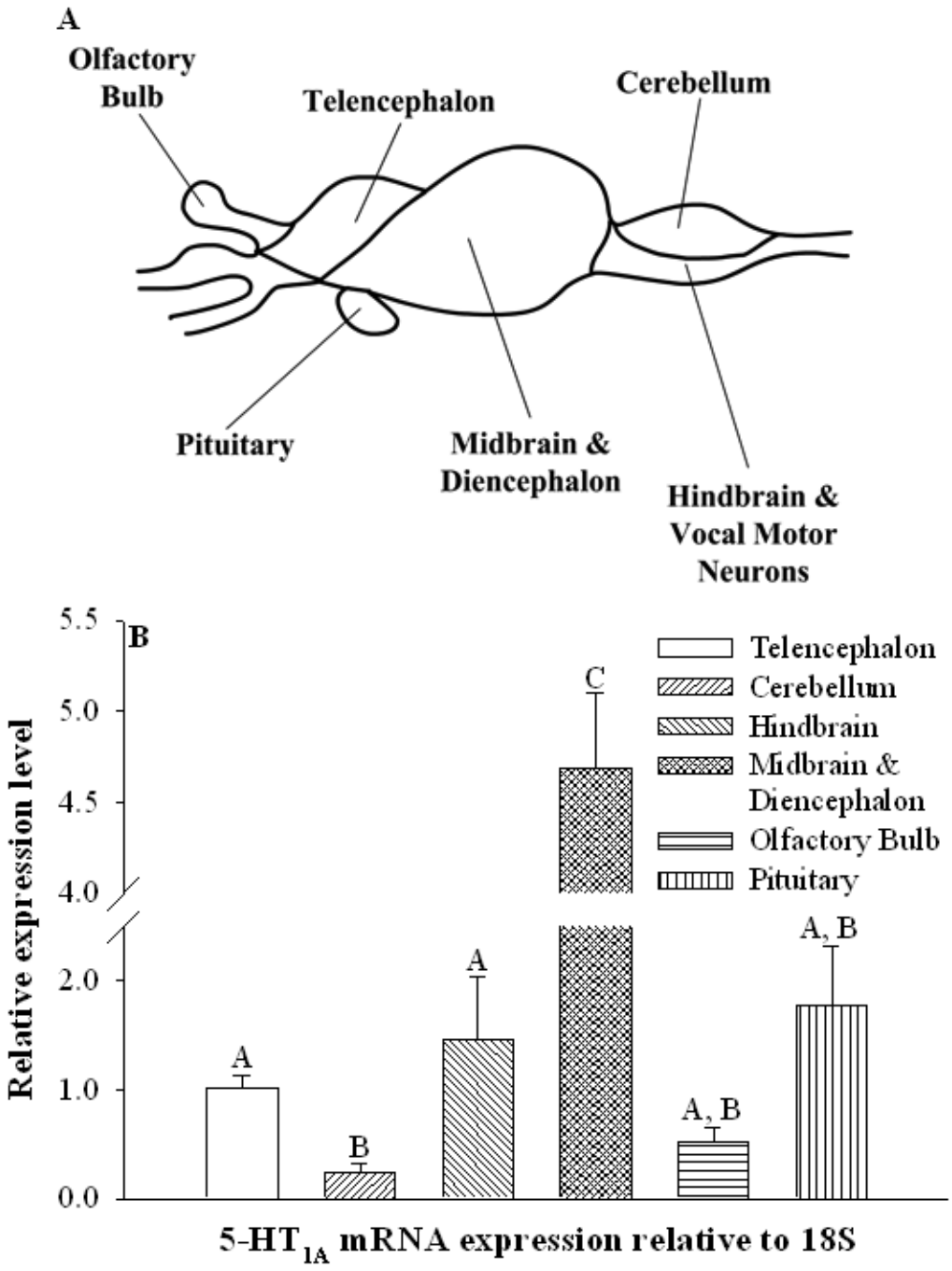

Figure 2.5. A. Schematic diagram depicting the way in which the brain was sectioned. Sectioning was intended to separate the brain into the following components: olfactory bulb, telencephalon, pituitary, midbrain & diencephalon, cerebellum, and the medulla & vocal motor neuron region. B. Relative quantity of 5-HT_{1A} mRNA expression present in various regions of the brain, with transcript levels normalized to the housekeeping gene 18S and quantities relative to the telencephalon. Values are means ± s.e.m., N=8. A different letter depicts significant difference ($p < 0.05$)

other tissues (e.g., the posterior intestine, ventral muscle, and rectum) (Fig. 2.4A). The heart, anterior intestine and kidney, which contain the interrenal cells that are a part of the HPI axis, all contained about 50-fold less 5-HT_{1A} mRNA compared to the whole brain (Fig. 2.4B). Different regions of the brain (Fig. 2.5A) expressed the receptor in varying amounts, with the midbrain and diencephalon region having the highest level of 5-HT_{1A} mRNA transcript (Fig. 2.5B). The midbrain and diencephalon region possessed at least twice as much transcript as other regions and up to 50-fold as much as the cerebellum, which had the lowest expression (Fig. 2.5B). In regards to the HPI axis, the midbrain and diencephalon region, which contains the hypothalamus and hippocampus, possessed about twice as much 5-HT_{1A} transcript as the pituitary alone.

Xenopus oocytes injected with toadfish 5-HT_{1A} cRNA showed a 1.9-fold increase in [^{3}H]5-HT binding compared to water-injected oocytes when incubated with 1.0 x 10^{-9} mol·L^{-1} of 5-HT alone (Fig. 2.6A). The non-specific component of binding was not subtracted in Figure 2.6A to demonstrate that the water-injected controls were binding a significantly lower amount of [^{3}H]5-HT than those injected with 5-HT_{1A} cRNA. For this reason, the binding of [^{3}H]5-HT appears significantly higher than in the subsequent binding experiments (below). A dose-dependent binding curve demonstrating saturation kinetics was established using [^{3}H]5-HT concentrations varying from 0-2 x 10^{-9} mol·L^{-1} ($P<0.01$, $R^2=0.98$; Fig. 1.6B), giving a B_{max} of 0.1192 x 10^{-15} mol·L^{-1} ($P < 0.01$) and a K_D of 4.998 x 10^{-10} mol·L^{-1} ($P < 0.01$). Due to the low K_D value ascertained from the 5-HT

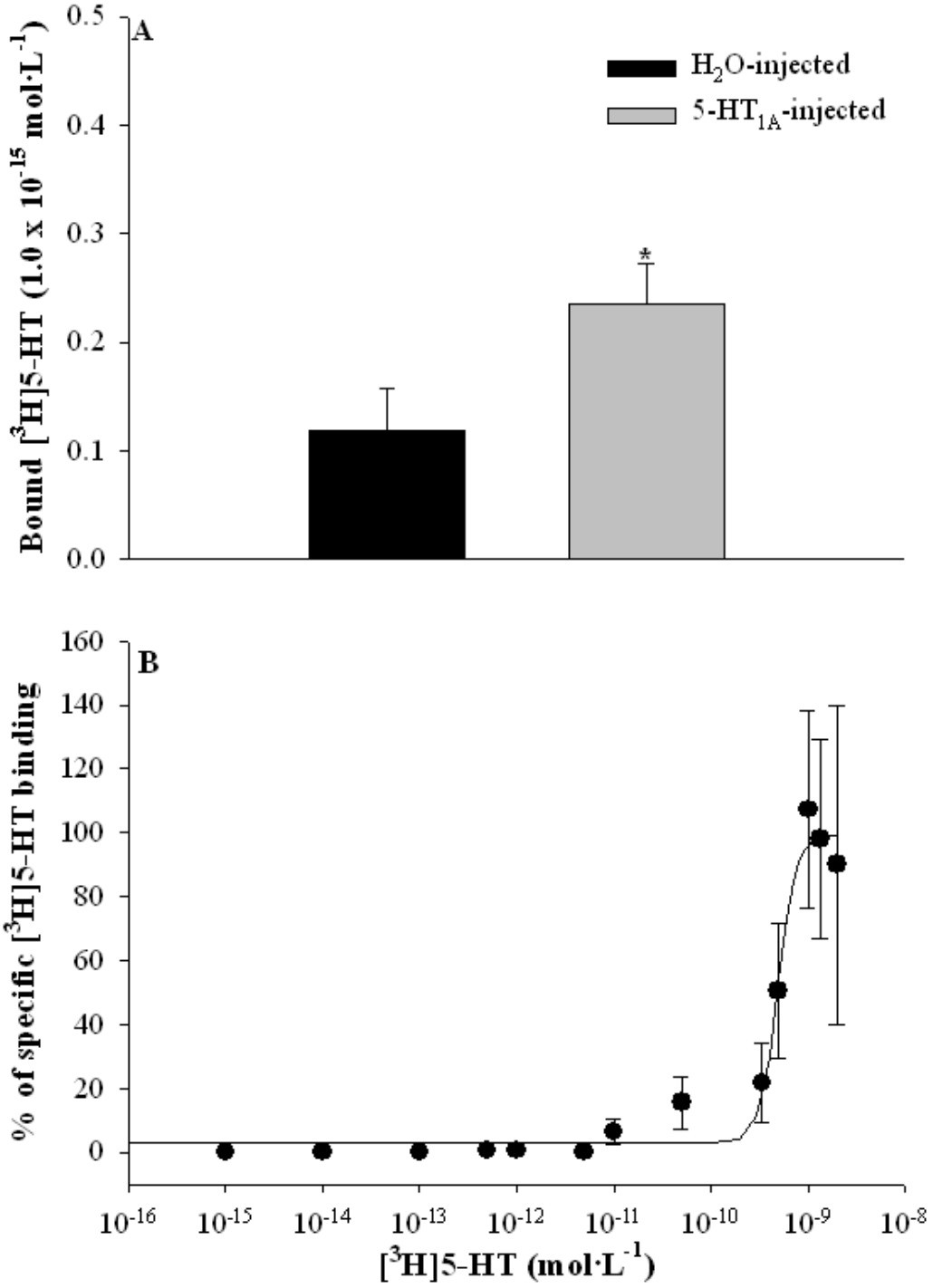

Figure 2.6. A. The amount of [^{3}H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT$_{1A}$ cRNA as compared to water-injected controls. Concentration of [^{3}H]5-HT was at 1.0 x 10^{-9} mol·L^{-1}. Values are means ± s.e.m., N=10, *p<0.05. B. The percent binding of [^{3}H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT$_{1A}$ cRNA when incubated in a particular concentration of [^{3}H]5-HT ranging from 0.0-2.0 x 10^{-9} mol·L^{-1}. Values are percentages of means relative to 0.0 mol·L^{-1} ± s.e.m. of the percentage, N=10.

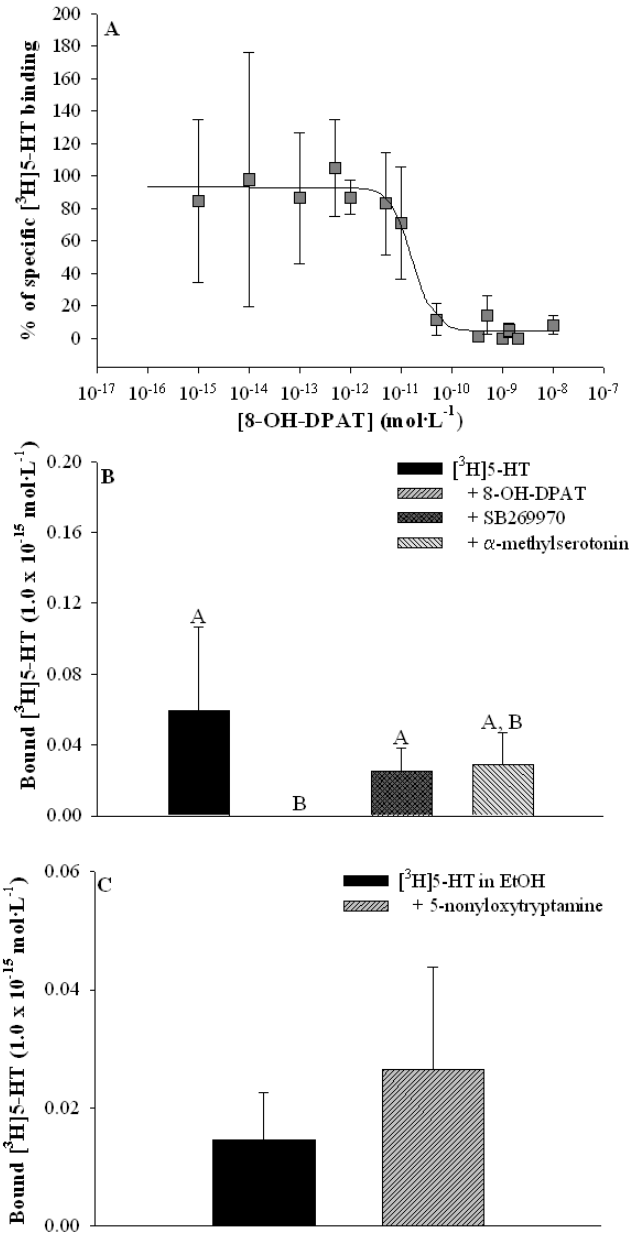

Figure 2.7. A. The percent of [^{3}H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT$_{1A}$ cRNA when incubated in the presence of 8-OH-DPAT ranging in concentration from 0.0-10.0 x 10^{-9} mol·L^{-1}. The concentration of [^{3}H]5-HT was 1.0 x 10^{-9} mol·L^{-1}. Values are percentages of means relative to binding at 2.0 nmol·L^{-1}, N=10. B. The amount of [^{3}H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT$_{1A}$ cRNA when incubated in the absence or presence of a mammalian 5-HT receptor agonist (8-OH-DPAT, specific for 5-HT$_{1A}$, and α-methylserotonin, specific for 5-HT$_2$) or antagonist (SB269970; specific for 5-HT$_7$). The concentration of [^{3}H]5-HT was 1.0 x 10^{-9} mol·L^{-1}. Values are means ± s.e.m., N=10. A different letter depicts a significant difference ($p<0.05$). C. The amount of [^{3}H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT$_{1A}$ cRNA when incubated in the absence or presence of the mammalian 5-HT$_{1B}$ receptor agonist 5-nonyloxytryptamine in 0.01% ethanol. 0.01% ethanol + [^{3}H]5-HT alone was used as a vehicle control. The concentration of [^{3}H]5-HT was 1.0 x 10^{-9} mol·L^{-1}. Values are means ± s.e.m., N=10. A different letter depicts a significant difference ($p<0.05$).

binding affinity curve, all subsequent competitive binding experiments used a fixed [^{3}H]5-HT concentration of 1.0 x 10^{-9} mol·L^{-1}. Binding of [^{3}H]5-HT by 5-HT_{1A}-injected oocytes was displaced by the mammalian 5-HT_{1A} receptor agonist, 8-OH-DPAT; a significant regression ($P < 0.01$; R^2=0.98) and an EC50 value of 2.02 x 10^{-11} mol·L^{-1} (P = 0.0006) was obtained for 8-OH-DPAT displacement over the range of 0.0-1.0 x 10^{-8} mol·L^{-1} 8-OH-DPAT (Fig. 2.7A). Displacement of [^{3}H]5-HT by agonists or an antagonist specific for other HTR subtypes was investigated using concentrations of pharmacological compounds close to the EC50 value established for 8-OH-DPAT (Fig. 2.7A), demonstrating significant displacement of [^{3}H]5-HT binding only in oocytes incubated with 8-OH-DPAT, an agonist specific for mammalian 5-HT_{1A} receptors, and not the pharmacological compounds specific for other HTR subtypes (Fig. 2.7B, 7C).

Intravenous injection with 8-OH-DPAT elicited a concentration-dependent increase in circulating levels of cortisol in the Gulf toadfish (Fig. 2.8A). When injected with 8-OH-DPAT at a dose of 16.25 mg·kg^{-1}·mL^{-1} saline, toadfish experienced a transient, though significant, increase in plasma cortisol levels within 0.25 h that returned

Table 2.2. Concentrations of circulating compounds in the plasma pre- and post-injection with 8-OH-DPAT or saline. Numbers reported as means ± s.e.m., N=6, no significant differences between times or treatments.

	Control			**8-OH-DPAT**		
Time (h)	**Glucose (mmol·L^{-1})**	**Protein (g·100mL^{-1})**	**Urea (mmol-N·L^{-1})**	**Glucose (mmol·L^{-1})**	**Protein (g·100mL^{-1})**	**Urea (mmol-N·L^{-1})**
0	1.98 ± 0.30	1.19 ± 0.15	9.78 ± 1.68	2.48 ± 0.46	1.27 ± 0.10	9.66 ± 1.65
0.5	1.73 ± 0.47	1.24 ± 0.19	6.18 ± 2.9	2.59 ± 1.46	0.86 ± 0.45	4.42 ± 1.14
1	1.62 ± 0.05	1.22 ± 0.05	8.56 ± 0.23	3.47 ± 1.63	1.13 ± 0.34	10.36 ± 3.20
2	2.04 ± 0.82	1.35 ± 0.11	9.88 ± 4.39	1.34 ± 0.34	1.22 ± 0.19	14.14 ± 4.22
4	0.63± 0.24	0.92 ± 0.29	15.59 ± 2.20	0.56 ± 0.10	1.30 ± 0.06	9.47 ± 2.18

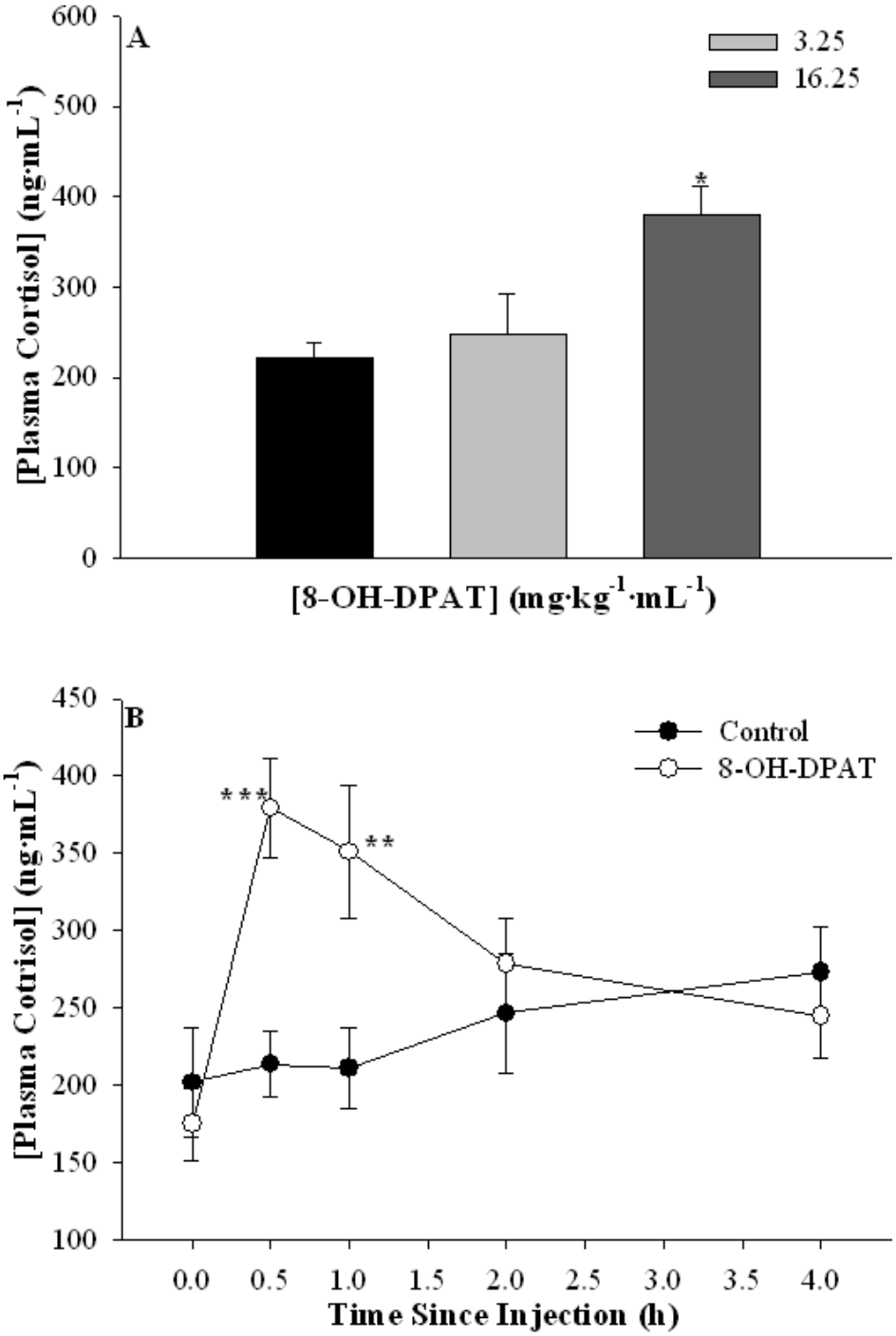

Figure 2.8. A. There is a concentration-dependent increase in circulating levels of cortisol in response to injection with 8-OH-DPAT. B. Changes in circulating levels of cortisol over time in response to injection with 16.25 mg·kg^{-1}·mL^{-1} saline 8-OH-DPAT. Controls are injected with saline alone. Values are means ± s.e.m., N=9, *p<0.05, **p<0.01, ***p<0.001.

to control levels within 4 hours (Fig. 2.8B). While there was a significant increase in plasma cortisol levels, this did not appear to result in a significant gluconeogenesis as circulating levels of glucose, protein or urea were not affected when compared to time zero or controls (Table 2.2).

Discussion

The phylogenetic analysis of the amino acid sequence supports a high degree of homology between the toadfish 5-HT_{1A} receptor and the human homologue, with the toadfish 5-HT_{1A} receptor falling out with the other 5-HT_{1A} amino acid sequences. Additionally, structural analysis of the amino acid sequence revealed a protein that contains 7 transmembrane domains, with an intracellular C terminus and an extracellular N terminus, which is congruent with the mammalian receptor (Raymond et al., 1999). When comparing the toadfish amino acid sequence to the human sequence, three substitutions become relevant in regards to binding and signaling: $Alanine^{356}$ in the toadfish is Threonine in humans, $Isoleucine^{364}$ is Methionine, and $Lysine^{366}$ becomes Arginine (Raymond et al., 1999). While the switch from Lysine in toadfish to Arginine in humans may not infer any change in binding, as both are α-amino acids and basic, the other two substitutions most likely confer a difference. Switching Isoleucine to Methionine within the G-protein binding motif may confer an increase in stability of the protein under certain coiling schemes (Garcia-Echeverria, 1997), and substituting Threonine for Alanine at the protein kinase C site may increase the number of sites available for phosphorylation or glycosylation (Brimer and Montie, 1998). Based on these inferences, most, if not all, of the function of the toadfish 5-HT_{1A} receptor should

be conserved; however, the change in amino acids may result in a change in the affinity constant or maximal binding of the receptor.

Consistent with tissue distribution studies investigating 5-HT_{1A} receptor mRNA expression in mammals (Engel et al., 2006; Kirchgessner et al., 1993; Raymond et al., 1993), the basic conclusion from the distribution of 5-HT_{1A} in toadfish is that the brain possesses up to 50-times more 5-HT_{1A} mRNA transcript than other tissues, including the kidney, which is where the interrenal cells are located, an integral part of the HPI axis. While there is significantly more 5-HT_{1A} mRNA present in the brain than in kidney, it is of note that there appears to be 5-HT_{1A} receptors present in all portions of the HPI axis. The reduced expression of 5-HT_{1A} receptors in the toadfish kidney is in agreement with mammalian studies that suggest that 5-HT_{1A} receptors do not play a role in stimulating the release of cortisol from the adrenal gland, the mammalian homologue to the teleost interrenal cells (Contesse et al., 1994; Lefebvre et al., 1992); however, as the whole kidney was used in the present study and not just isolated interrenal cells, the relative mRNA expression of the toadfish 5-HT1A by the interrenal cells is likely underestimated. Furthermore, renal 5-HT_{1A} protein function was not evaluated in the present study, and since mRNA expression does not always represent protein activity (McDonald et al., 2009), it cannot be concluded that 5-HT_{1A} receptors in the interrenal cells are not playing a substantial role. Surprisingly, the toadfish swim bladder and swim bladder muscle also possess high amounts of 5-HT_{1A} receptor. The toadfish is so named for the croaking noises it emits when agitated, but the males are also able to produce a boatwhistle call when attracting mates to potential nesting sites (Goodson and Bass, 2002; Serafy et al., 1997). Sound production occurs as a result of the swim bladder

muscle rapidly contracting against the swim bladder membrane, a movement that is controlled by the vocal motor neurons (Goodson and Bass, 2002), located in the hindbrain where moderate amounts of 5-HT_{1A} receptor is expressed. Recent work investigating 5-HT_{1A} receptor function in mammals and fish has focused on the involvement of the receptor in reproduction and associated behaviors (Smith and Combs, 2008; Wada et al., 2006), supporting a potential role of the 5-HT_{1A} receptor in toadfish, potentially in the modulation of sound production that is part of their courting behavior.

Studies conducted in rats and humans have found that the 5-HT_{1A} receptor is widely distributed throughout the brain, though the amount of mRNA and/or binding activity varies greatly depending on the region (Chalmers and Watson, 1991; Pompeiano et al., 1992). In mammals, particularly high expression of 5-HT_{1A} is found within the hippocampus, pituitary, and raphe nuclei whereas the cerebellum, amygdala, corpus striatum and globus pallidus have especially low levels (Kumar and Mann, 2007; Passchier et al., 2000). In terms of function, the hippocampus is important in providing negative feedback on the HPA axis while the pituitary is paramount to most endocrine functions, including the regulation of the HPA axis. The raphe nuclei is a major source of brain 5-HT and contains the somatodendritic 5-HT_{1A} autoreceptors, which play a role in controlling the release of 5-HT in other areas of the CNS. Distribution of 5-HT_{1A} transcript throughout the brain varied in toadfish greatly, though the expression pattern is congruent with the mammalian studies. The toadfish midbrain and diencephalon section, which includes the pallial region (analogous to the hippocampus in higher vertebrates) and the rostral raphe nuclei, contained a significantly higher level of 5-HT_{1A} mRNA transcript than all other regions. Given the relatively high conservation of the

serotonergic system in vertebrates, it is likely that the high expression of 5-HT_{1A} receptors found in this region is due to the rostral raphe nuclei and pallial region, as the 5-HT_{1A} receptor is present only in low levels in the hypothalamus of humans and other mammals (Kumar and Mann, 2007; Passchier et al., 2000). The relatively high amounts of 5-HT_{1A} mRNA transcript in the toadfish pituitary is consistent to what is found in mammals (Chalmers and Watson, 1991; Kumar and Mann, 2007; Lopez et al., 1998; Passchier et al., 2000), not surprising if the role of 5-HT in the regulation of the stress response is conserved across taxa.

Supporting the apparent conservation of function across taxa, 5-HT_{1A}-injected *Xenopus* oocytes incubated with 1.35 x 10^{-9} mol·L^{-1} [^{3}H]5-HT, the predicted K_D for the population of 5-HT receptors in toadfish brain determined using a whole-brain homogenate preparation (LR Medeiros and MD McDonald, unpublished observation), had significantly higher [^{3}H]5-HT binding compared to the water-injected oocytes. Furthermore, 5-HT_{1A}-injected *Xenopus* oocytes incubated with [^{3}H]5-HT and equal amounts of 8-OH-DPAT exhibited significantly lower binding as compared to those incubated with [^{3}H]5-HT alone, indicating that the toadfish 5-HT_{1A} receptor binds both 5-HT and the mammalian 5-HT_{1A} agonist, 8-OH-DPAT. That 8-OH-DPAT was able to completely eliminate 5-HT binding when present in equal concentrations suggests that the toadfish 5-HT_{1A} receptor has a higher affinity for 8-OH-DPAT than for 5-HT, a trend that is also seen in the mammalian 5-HT_{1A} receptor (Dompert et al., 1985; Hoyer et al., 1985; Peroutka, 1986). This assumption was confirmed when performing the 5-HT and 8-OH-DPAT dose-dependent binding curve, when the K_D for 5-HT binding (4.998 x 10^{-10} mol·L^{-1}) was determined to be about half an order of magnitude higher than the EC50

determined for 8-OH-DPAT (2.02 x 10^{-11} mol·L^{-1}). This is in accordance with the findings observed in studies on other species of fish as well as mammals (Winberg and Nilsson, 1996; Zifa and Fillion, 1992). That all other subtypes' agonists and antagonists were unable to abolish the binding of 5-HT further supports that the toadfish 5-HT_{1A} receptor has very similar, if not identical, binding sites as the mammalian counterpart. Interestingly, the concentration of 8-OH-DPAT used in this set of experiments (1.8 x 10^{-11} mol·L^{-1}; Fig. 7B) was able to completely eliminate binding of [^{3}H]5-HT. This concentration is slightly lower than the established EC50 (Fig. 7B *cf.* Fig. 7A); the regression obtained from the EC50 curve predicts that about 55% of binding would still be present at this concentration of 8-OH-DPAT. The most likely explanation for the anomaly is that the slope of the curve at this concentration is extremely steep, and therefore contains more inherent variation at these concentrations than at either end of the range.

The similarity between the toadfish 5-HT_{1A} receptor and mammalian homologue in terms of amino acid sequence, mRNA distribution within the HPI axis and pharmacological sensitivity was further supported by functional similarity on the whole animal level. Injection with the mammalian 5-HT_{1A} agonist 8-OH-DPAT caused a significant increase in plasma cortisol levels that returned to control levels within 4 hours. The endocrine response to 8-OH-DPAT injection, combined with the *Xenopus* oocyte data, indicates a specific, potent effect mediated by the 5-HT_{1A} receptor in the Gulf toadfish. The timing of the response is consistent with other teleost fish (Höglund et al., 2002; Winberg et al., 1997; Zifa and Fillion, 1992). For example, rainbow trout (*Oncorhynchus mykiss*) injected with a 0.040 mg·kg^{-1} dose of 8-OH-DPAT experienced a

significant increase in plasma cortisol levels within 30 min that returned to control levels after 4 h (Winberg et al., 1997). The higher dose of 8-OH-DPAT needed to elicit a response in toadfish (16.25 $mg \cdot kg^{-1}$) may be due to differences in ambient water temperature (see Sundin et al., 1998) or a species-specific response to 8-OH-DPAT. In any case, the observed increase in and subsequent metabolism of plasma cortisol in toadfish in response to 8-OH-DPAT is similar to what is observed in mammals when injected with the same compound (Zifa and Fillion, 1992), indicating that, in addition to a molecular and pharmacological consistency between the 5-HT_{1A} receptor of mammals and toadfish, there is also a functional similarity in terms of its role in the regulation of the stress response. Based on our results from the *in vitro* oocyte binding assays, it is unlikely that the increase in circulating levels of cortisol, due to activation of 5-HT_{1A}, could be mediated by another 5-HT receptor agonist or antagonist. However, we have not eliminated the possibility that 8-OH-DPAT may be activating other receptors in this non-mammalian system that are involved in stimulating cortisol release (i.e., 5-HT_4 receptors; Lefebvre et al., 1992; Contesse et al., 1994), which could be contributing to the observed *in vivo* response.

This investigation provides the first look at 5-HT_{1A} receptor molecular characteristics, tissue distribution, pharmacology and neuroendocrine function in a single teleost fish species. The findings suggest a high conservation of the 5-HT_{1A} receptor amongst vertebrates and validate the Gulf toadfish as a potential model organism for the study of vertebrate 5-HT_{1A} receptor function. Due to the emerging evidence concerning the presence and opposing functionality of the two 5-HT_{1A} receptor populations (somatodendritic autoreceptors in the raphe nucleus and postsynaptic receptors elsewhere

in the brain and periphery; Lanfumey and Hamon, 2000), future investigations would benefit from utilizing recently developed agonists that differentiate between the two populations in order to determine a clear role for each in terms of HPI axis regulation. Only by increasing our knowledge of 5-HT_{1A} receptor function can we begin to understand the more complicated physiological and behavioral systems with which it is involved.

CHAPTER 3
CORTISOL-MEDIATED DOWNREGULATION OF THE SEROTONIN RECEPTOR SUBTYPE 1A IN THE GULF TOADFISH, *OPSANUS BETA*

Background & significance

The serotonin (5-hydroxytryptamine; 5-HT) 1A (5-HT_{1A}) receptor is one of the most abundant 5-HT receptor subtypes in the mammalian brain and, being the first 5-HT receptor cloned, has been the most studied (Albert et al., 1990; Laaris et al., 1995). This G-protein-coupled receptor employs a G_i/G_0 transduction system that primarily decreases adenylate cyclase formation and/or increases K^+ conductance (Reviewed by Laaris et al., 1995; Calogero et al., 1990). These mechanisms are postulated to inhibit firing of the postsynaptic cell, and it has been observed that 5-HT, mediated by the 5-HT_{1A} receptor, exerts a predominantly inhibitory effect on neuron firing rate in many areas of the brain (Clark et al., 1987; Araneda and Andrade, 1991; Kow et al., 1992). Agonists of the mammalian 5-HT_{1A} receptor, such as 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT), have been found to elevate plasma corticosteroid levels in mammals (Dinan, 1996), implicating the 5-HT_{1A} receptor as an important activator of the mammalian hypothalamic-pituitary-adrenal (HPA) axis, the primary endocrine axis involved in the stress response. Depending on the location of the receptor, activation of the HPA axis has been shown to be achieved when 8-OH-DPAT is applied directly to the rat hypothalamus, stimulating the release of CRH, and when applied directly to the pituitary, triggering the secretion of ACTH (Calogero et al., 1990)

The 5-HT_{1A} receptor has been fully cloned in the Gulf toadfish, *Opsanus beta*, being nearly 70% identical to the human gene (Medeiros et al., 2010). The receptor has also been cloned in other species of fish (Wang and Tsai, 2006; Yamaguchi and Brenner, 1997), all showing the same high levels of similarity to the human homologue. In the Gulf toadfish, expression of 5-HT_{1A} mRNA is concentrated within the brain and pituitary, with nearly undetectable transcript levels in the kidney (Medeiros et al., 2010). In a previous study, we have shown that the pharmacological characteristics of the Gulf toadfish 5-HT_{1A} receptor are similar to those observed in mammals (Barnes, 1999; Peroutka and Howell, 1997) and other teleosts (Winberg and Nilsson, 1996), with similar binding affinities (K_D values) for both 5-HT and 8-OH-DPAT (Medeiros et al., 2010). The Gulf toadfish 5-HT_{1A} receptor exhibits similar sensitivity, or lack thereof, to other mammalian agonists and antagonists; 5-HT binding to the toadfish 5-HT_{1A} receptor was not competitively inhibited by ligands specific for other 5-HT receptors (Medeiros et al., 2010). Furthermore, similar to its actions in mammals, 8-OH-DPAT has been shown to stimulate the release of cortisol in the Gulf toadfish, *Opsanus beta* (Medeiros et al., 2010) as well as the rainbow trout, *Oncorhynchus mykiss* (Winberg et al., 1997), with an effective dose similar to binding affinities measured in mammals (Peroutka and Howell, 1997). Work done on teleost fish investigating the interaction between 5-HT, the 5-HT_{1A} receptor and the hypothalamic-pituitary-interrenal (HPI) axis, the teleost homologue of the HPA axis, has mostly focused on how these systems work together during social interactions (Winberg and Nilsson, 1993; Larson et al., 2003; Clotfelter et al., 2007; McDonald et al., 2011). Specifically, social stressors, such as subordination, result in an elevation in brain 5-HT turnover as indicated by brain 5-hydroxyindoleacetic acid (5-

HIAA; the major 5-HT metabolite) concentrations and 5-HIAA:5-HT ratios (Winberg and Nilsson, 1993). At the same time, socially subordinate fish display elevated plasma cortisol levels (Ejike and Schreck, 1980) and increased interrenal cell size, suggesting a chronic activation of the HPI axis (Noakes and Leatherland, 1977).

Thus, it is clear that 5-HT stimulates glucocorticoid secretion via the 5-HT_{1A} receptor in both mammals and teleost fish. In mammals, there is also evidence for a homeostatic role for glucocorticoids in maintaining 5-HT_{1A} receptor level and function. Whereas adrenalectomy, which removes background levels of glucocorticoids, increases [^{3}H]8-OH-DPAT binding in the CA2 region of the hippocampus (Mendelson and McEwen, 1991), there is also evidence for the presence of a negative feedback loop whereby highly elevated circulating levels of glucocorticoids inhibit 5-HT_{1A} receptor activity (Zhong and Ciaranello, 1995), in addition to directly inhibiting the release of ACTH and CRH (Canny et al., 1989; Delbende et al., 1992). In mammals, a decreased expression of 5-HT_{1A} mRNA in response to elevated glucocorticoids is correlated with a decrease in binding (Laaris et al., 1995) and function (Porter et al., 1998), which is thought to be mediated via the glucocorticoid receptor (GR) and not the mineralocorticoid receptor (MR; de Kloet et al., 2009; Meijer and de Kloet, 1994). Furthermore, elevated glucocorticoids have been shown to downregulate 5-HT_{1A} function independent of transcript level or protein activity (Van de Kar et al., 1998).

Since mounting evidence suggests that the serotonergic system appears to be highly conserved between mammals and teleosts, it is likely that chronic elevations in glucocorticoids may also suppress the 5-HT_{1A} receptor in teleosts as it does in mammals (Chalmers et al., 1993; Meijer and de Kloet, 1994). To investigate the possibility of such

a reciprocal relationship between the HPI axis and the 5-HT_{1A} receptor in teleosts, this study explored whether the Gulf toadfish 5-HT_{1A} receptor was sensitive to cortisol at the level of mRNA expression, protein binding, or whole animal function. Based on the mammalian literature and Medeiros et al. (2010), which demonstrated a high degree of molecular and functional similarity between the Gulf toadfish and the mammalian 5-HT_{1A} receptor, we hypothesized that 5-HT_{1A} receptor mRNA expression, protein binding and function would decrease in response to endogenous cortisol elevation due to crowding. To investigate the role of GRs or MRs in mediating the effects of cortisol, RU486 and spironolactone were used, which inhibit cortisol from binding to GRs and MRs, respectively.

Materials and Methods

Experimental Animals. Gulf toadfish (*Opsanus beta*) were captured via roller trawl by commercial shrimpers in Biscayne Bay, Florida in the summer of 2010. The toadfish were then immediately transferred to the laboratory where they were held for up to one month. Fish were treated with a dose of malachite green (final concentration 0.05 $mg \cdot L^{-1}$) in formalin (15 $mg \cdot L^{-1}$) (AquaVet) on the day of transfer to the laboratory in order to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993). The fish were kept in 50L glass aquaria with flowing, aerated seawater in relatively uncrowded conditions (10 g $fish \cdot L^{-1}$) (Walsh et al., 1994). The water temperature was kept between 18 and 21ºC. Fish were fed weekly with shrimp. All protocols were approved by the University of Miami IACUC.

Experimental Treatments

Series 1: Endogenous plasma cortisol elevation

Uncrowded toadfish (43.7 ± 3.4 g, N=16) were placed individually into minnow traps and separated into 4 outdoor 6000 L mesocosm tanks (4 fish per tank) seeded with the seagrass, *Thalassia testudinu ,* emulating the natural environment of Gulf toadfish (Serafy et al., 1997). In addition, two separate groups of toadfish were crowded (10 fish per 10 L water) in the laboratory and left for one week before collection, with the smallest 8 fish sampled each time (52.2 ± 3.0 g, N=16) (to be consistent in terms of social status). To decrease the chance of handling stress confounding results, blood samples were drawn immediately after removing the fish from the water *via* ventral caudal puncture using a 23G needle attached to a disposable syringe as described by Medeiros et al. (2010). Each fish was sampled within a 5 minute period; this time period is short enough so that plasma cortisol levels are indicative of basal levels and not the result of sampling (Medeiros et al. 2010). Collected samples were centrifuged at 16,000 *g* for 10 minutes and the plasma decanted. Plasma from each sample was flash frozen in liquid nitrogen (N_2), and then stored at -80°C until used for the evaluation of circulating levels of cortisol. Brain tissue was collected from fish first anesthetized with a lethal dose of MS-222 (3 $g \cdot L^{-1}$; Finquel). Brain samples were either frozen immediately in liquid N_2, and stored at –80°C to be used in quantitative real-time PCR (qPCR) or used immediately in a whole brain homogenate binding assay.

Series 2: Pharmacological treatment with RU486 and spironolactone.

The glucocorticoid receptor antagonist, RU486 (mifepristone; Sigma Aldrich), and mineralocorticoid receptor antagonist, spironolactone (Sigma Aldrich), were used to determine the involvement of the GR and MR, respectively, in regulating 5-HT_{1A} mRNA expression, binding and function in crowded toadfish. For each of the 3 experimental protocols (see *Experimental Protocols* below), a subset of 10 fish were subjected to one week of crowding in a 10 L tub in the laboratory, out of those 10 fish, N = 8 toadfish were anesthetized (MS 222; 1 $g \cdot L^{-1}$) prior to being surgically implanted with an intraperitoneal (IP) catheter (Clay-Adams PE160, Franklin Lakes, NJ, USA) filled with peanut oil as described by McDonald and Walsh (2004). The IP catheter was inserted through a small ventral incision and threaded approximately 4 cm inside the body cavity. After surgery, the fish were placed in individual 2 L tubs and left to recover undisturbed for 24 h. After the recovery period, fish were injected intraperitoneally with either 10 $mg \cdot 100\ g^{-1}$ RU486 in 1 $ml \cdot 100\ g\ fish^{-1}$ peanut oil followed by 0.3 ml of peanut oil (mean mass 58.7 ± 3.7 g, $N = 24$), 5 $mg \cdot 100\ g\ fish^{-1}$ spironolactone in 1 $ml \cdot 100\ g^{-1}$ peanut oil followed by a push of 0.3 ml of peanut oil to clear the catheter (mean mass 46.0 ± 4.1 g, $N = 24$), 10 $mg \cdot 100\ g\ fish^{-1}$ RU486 mixed with 5 $mg \cdot 100\ g^{-1}$ spironolactone in 1 $ml \cdot 100\ g\ fish^{-1}$ peanut oil followed by a push of 0.3 ml of peanut oil (mean mass 54.9 ± 2.3 g, $N = 8$) or peanut oil alone (1 $ml \cdot 100\ g\ fish^{-1}$ peanut oil followed by 0.3 mL of peanut oil) (mean mass 49.6 ± 2.1 g, $N = 56$). The doses used were based on results from preliminary experiments as well as previous studies on fish (McDonald et al., 2004; Rodela et al., 2009). Injections were repeated every 12h and after 48h of treatment, a blood sample was taken via a caudal puncture. Blood samples were centrifuged at 16,000

g for 10 min and the plasma decanted. Plasma samples were frozen immediately in liquid N_2 and stored as described above. Eight fish from the RU486, spironolactone and peanut oil treatments were used in an 8-OH-DPAT challenge as described below. The remaining fish were anesthetized with a lethal dose of MS-222 (3 $g \cdot L^{-1}$) and brain tissue was dissected; half of the brain samples (N = 8) were used immediately in whole brain homogenate binding assays. The remaining brains (N = 8) were immediately frozen in liquid N_2 to be used for qPCR analysis. The fish from the RU486 + spironolactone treatment group were only used in an 8-OH-DPAT challenge (as described below).

Series 3: Exogenous cortisol loading with coconut oil implants

To determine the effect of cortisol alone on the functional response of the 5-HT_{1A} receptor without the social stress of crowding, indwelling caudal venous/arterial catheters were surgically inserted into anesthetized toadfish as described previously by McDonald et al. (2000). While still under anesthetic, fish were also injected with either 1 mL coconut oil·100 g $fish^{-1}$ alone (vehicle fish [66.9 ± 3.6 (6) g]) or with cortisol (11β,17α,21-Trihydroxy-4-pregnene-3,20-dione 21-hemisuccinate sodium salt; Sigma Aldrich) at a dose of 11.5 mg cortisol·mL coconut oil·100 g $fish^{-1}$ (cortisol-implanted fish [69.0 ± 5.0 (6) g]) as described by Vijayan et al. (1994) and Morando et al. (2009). Intraperitoneal implantation of cortisol using coconut oil as a vehicle has been shown to produce a slow release of cortisol into the circulation (Vijayan and Leatherland, 1989). After injection with the warmed coconut oil, an ice pack was placed against the site of injection to facilitate the solidification of the implant. Following the surgery, fish were placed in individual 2 L tubs and left undisturbed for 48h, after which time they

were used to assess 5-HT_{1A} receptor function with an 8-OH-DPAT challenge (see below).

Experimental Protocols

RNA isolation, cDNA synthesis and quantitative (real-time) PCR (qPCR) analysis. Total RNA was isolated from brain following the protocol provided with the Trizol reagent (Invitrogen). Total RNA was subsequently treated with DNAse I to remove potential residual genomic DNA according to the protocol provided with the TurboDNA-free kit (Ambion). RNA integrity was analyzed *via* gel electrophoresis before proceeding to cDNA synthesis. For use in qPCR, cDNA was synthesized with random hexamer primers from 1 μg of DNase I-treated total RNA according to the protocol provided with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), and 'no reverse transcriptase' controls were routinely performed as an additional quality check. A ten-fold dilution of the product was made up using double-deionized RNA- and DNA-free water (Sigma Aldrich). Primer sequences for the toadfish 5-HT_{1A} receptor (gene of interest), and the normalizing gene, 18S, were as described in Medeiros et al. (2010). Quantitative PCR was performed using an Mx3005P Multiple Quantitative PCR system (Stratagene) using the protocol described by Medeiros et al. (2010). The standard curves of the gene of interest and normalizers genes in this study gave PCR efficiencies of 101.9% (5-HT_{1A}; R^2=0.99) and 102.3% (18S; R^2=1.0). To confirm that there was no contamination or primer dimer formation contributing to the fluorescence measured, 'no reverse transcriptase' and 'no template controls' were performed.

Binding assays using whole brain homogenate. Brains were prepared for the binding assay using the protocol outlined by Bennett and Snyder (1976) and Winberg and Nilsson (1996). Briefly, toadfish brains were removed and placed in ice-cold 50mmol·L^{-1} Tris–HCl (pH 7.4) until homogenization. Combined brains from each treatment (uncrowded, N = 8; crowded, N = 8; vehicle-treated, N = 8; RU486-treated, N = 8; or spironolactone-treated, N = 8) were homogenized in 10mL of Tris–HCl using a glass Potter–Elvehjem homogenizer and then centrifuged at 45 000 *g* for 12 min. The supernatant was discarded and the pellet was resuspended in 10 mL of Tris–HCl buffer and incubated at 25°C for 30 min before a second centrifugation at 45 000 *g* for 12 min. The pellet was again resuspended in 10 mL of Tris–HCl and centrifuged at 45 000 *g* for 12 min. The final pellet was resuspended in 10 mL of Tris–HCl buffer (pH 7.4) containing: 10 mmol·L^{-1} pargyline, 4 mmol·L^{-1} $CaCl_2$ and 0.1% ascorbic acid. The suspension was then immediately used in the following binding assay: 0.8 mL of tissue suspension (approximately 0.5 mg protein·mL^{-1}) + 0.1 mL of buffer + a known concentration (5–100 nmol L^{-1}) of [^{3}H]8-OH-DPAT (~170.4 Ci·$mmol^{-1}$; American Radiolabeled Chemicals, Inc.) in 0.1 mL of buffer, resulting in a 10-fold dilution of 8-OH-DPAT concentrations (*i.e.,* actual concentrations in the 1 ml solution were 0.5–10.0 nmol L^{-1}). Following a 45 min incubation period at 25°C, the assay mixture was vacuum filtered through Whatman GF/A glass fibre filters using a 3-place Pall filter funnel manifold (Pall Life Sciences), and rinsed three times in 5 mL of ice-cold Tris–HCl buffer. Radioactivity was measured by liquid scintillation counting (Tri-Carb 2100TR Liquid Scintillation Counter, Packard) in 10 mL of EcoLume™ liquid scintillation fluid (MP Biomedicals). Non-specific binding was accounted for by subtracting the counts from the filters incubated with

[^{3}H]8-OH-DPAT alone from the counts from the filters incubated with brain homogenate + [^{3}H]8-OH-DPAT.

8-OH-DPAT challenge. The function of the 5-HT_{1A} receptor *in vivo* was assessed with an 8-OH-DPAT challenge. Indwelling caudal vein catheters were inserted into a subset of fish from all treatments of *Series 2* and *3* as described by McDonald et al. (2000). Immediately after IP catheter implantation (*Series 2*) or IP cortisol implantation (*Series 3*), the caudal vertebrae were exposed by a 1.5–2.0 cm lateral incision between the epaxial and hypaxial muscle masses. The haemal arch was cannulated with PE50 tubing filled with heparanized saline (50 i.u mL^{-1} sodium heparin; Sigma). A heat-flared PE160 sleeve was then glued in place with cyanoacrylate tissue cement and sutured at the site of exit to secure the catheter. To prevent infection, the wound was then covered with a generous dusting of oxytetracycline powder and sutured securely with 3-0 silk. Following surgery, fish were allowed to recover for a full day. An initial 300 µL blood sample was drawn via the caudal vein catheter immediately before intravenous injection 8-OH-DPAT at a dose of 1.63 mg·100 g $fish^{-1}$ in 0.1 ml saline·$100g^{-1}$. This dose has been established by Medeiros et al. (2010) to cause a significant increase in plasma cortisol levels within 30 min in Gulf toadfish. After 30 min, a final (300µL) blood sample was drawn via the caudal catheter. Blood samples were centrifuged and stored as described above for later analysis of cortisol concentration.

Analytical Techniques

Plasma cortisol was quantified using the MP Biomedical ^{125}I RIA kit, with the standards diluted by half so that protein concentrations were within the range measured in toadfish. Protein concentrations of the binding assay tissue suspensions were assayed using the Bradford Reagent Assay (Sigma Aldrich).

Statistics

Unless otherwise noted, analyses were conducted using SigmaStat and/or SigmaPlot (SPSS, Inc.). Data are given as means ± 1 s.e.m., and N = the number of fish in each assay. Differences between two means were determined using either the Student's unpaired two-tailed *t*-test ($p<0.05$) or, if data were not normally distributed even after log transformation, the Mann-Whitney rank sum test ($p<0.05$). To determine differences amongst treatments, an ANOVA was performed, with a Bonferroni post-hoc test following when a significant difference was found ($p<0.05$). Sigmoidal dose-response curves were established using Prism (version 5.0; GraphPad Software, Inc). Curve parameters were compared using a residual sum-of-squares F-test (version 5.0; GraphPad Software, Inc).

Results

The effect of endogenous cortisol elevation on brain 5-HT$_{1A}$ mRNA expression and binding kinetics was evaluated by comparing fish that were stressed by crowding to fish that were held in uncrowded conditions. Plasma cortisol levels were, on average,

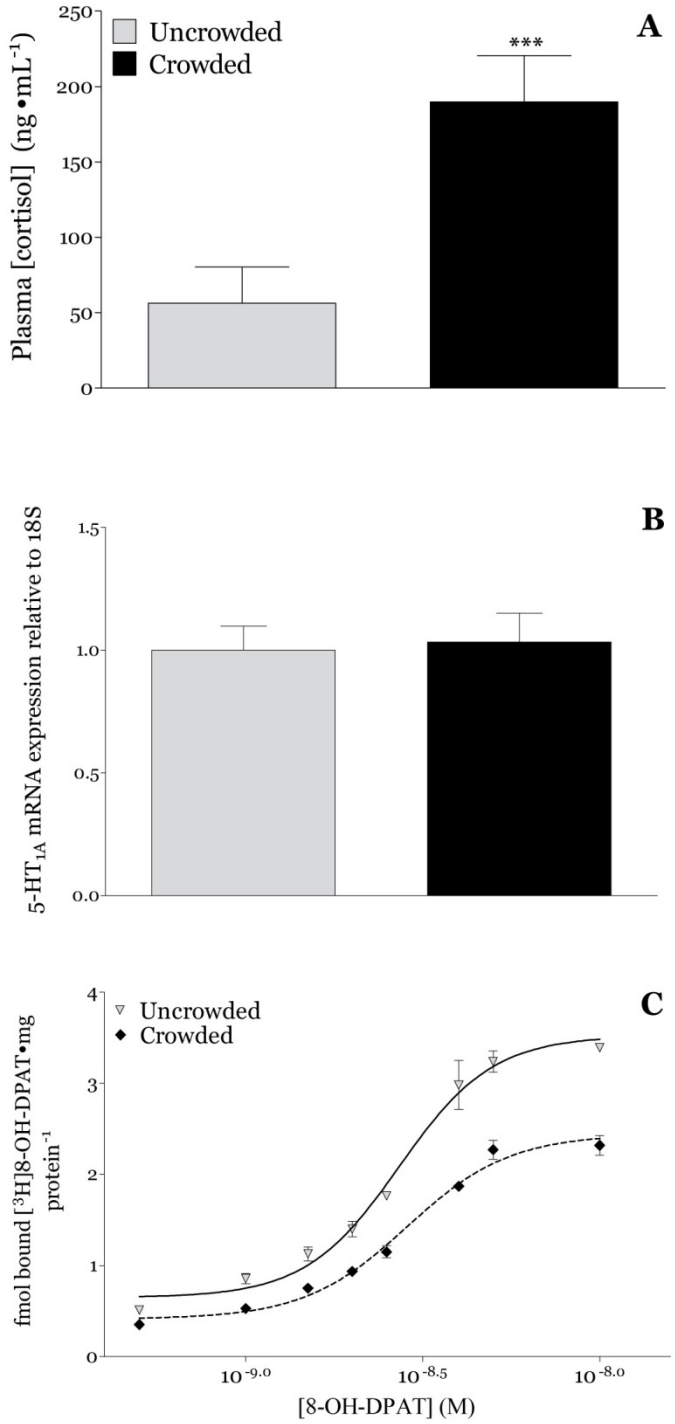

Figure 3.1. A. Crowded toadfish had nearly 4-fold higher circulating cortisol concentrations compared to uncrowded controls ($p < 0.001$). B. High cortisol levels in crowded toadfish were associated with no change in 5-HT_{1A} mRNA expression compared to uncrowded fish. C. Crowded fish had significantly less 5-HT_{1A} receptor protein available for binding to [^{3}H]8-OH-DPAT (B_{max} uncrowded: $3.52 \pm 0.16 \times 10^{-15}$ mol·mg protein^{-1} and crowded: $2.44 \pm 0.09 \times 10^{-15}$ mol·mg protein^{-1}; $p < 0.05$); however, binding affinity did not change (uncrowded = $2.71 \pm 1.06 \times 10^{-9}$ mol·L^{-1}, crowded = $2.86 \pm 1.05 \times 10^{-9}$ mol·L^{-1}). Data are means ± s.e.m; N = 8; *** $p < 0.001$. For binding kinetics, data presented is from 8 combined brains per treatment group, s.e.m. represents N = 3 technical replicates.

four-times higher in crowded toadfish compared to the uncrowded controls ($p < 0.001$; Fig. 3.1A). Brain 5-HT_{1A} transcript levels in crowded and uncrowded Gulf toadfish were not significantly different ($p = 0.833$; Fig. 3.1B). In contrast, 5-HT_{1A} receptor binding capacity (B_{max}) was significantly lower in crowded toadfish ($p < 0.05$; Fig. 3.1C) than the uncrowded fish ($2.44 \pm 0.09 \times 10^{-15}$ mol·mg protein^{-1} compared to $3.52 \pm 0.16 \times 10^{-15}$ mol·mg protein^{-1}, respectively; Fig. 3.1C). However, the K_D did not vary significantly between the two groups of fish (crowded $K_D = 2.86 \pm 1.05 \times 10^{-9}$ mol·L^{-1} and uncrowded $K_D = 2.71 \pm 1.06 \times 10^{-9}$ mol·L^{-1}).

The role of the glucocorticoid receptor (GR) in mediating the effects of cortisol on the 5-HT_{1A} receptor was investigated by using the GR antagonist, RU486, which interferes with the physiological response to cortisol. Treating crowded fish with RU486 caused a significant ($p < 0.001$) increase in circulating cortisol levels, with twice the amount of plasma cortisol in the RU486-treated fish compared to vehicle-injected control fish (Fig. 3.2A). Despite higher cortisol levels, RU486-treated fish had twice the amount of 5-HT_{1A} mRNA transcript found in the vehicle-injected controls ($p < 0.001$; Fig. 3.2B). Along these same lines, the RU486-injected treatment had a significantly higher B_{max} ($p < 0.05$; Fig. 3.2C) compared to the vehicle-injected controls ($0.87 \pm 0.03 \times 10^{-15}$ mol·mg protein^{-1} and $0.49 \pm 0.023 \times 10^{-15}$ mol·mg protein^{-1}, respectively) although, similar to the naturally manipulated treatments, K_D did not vary significantly between the groups ($1.34 \pm 1.04 \times 10^{-9}$ mol·L^{-1} and $1.21 \pm 1.07 \times 10^{-9}$ mol·L^{-1}, respectively).

The role of the MR in mediating the effects of cortisol on the 5-HT_{1A} receptor was investigated by using the MR antagonist, spironolactone, which interferes with the

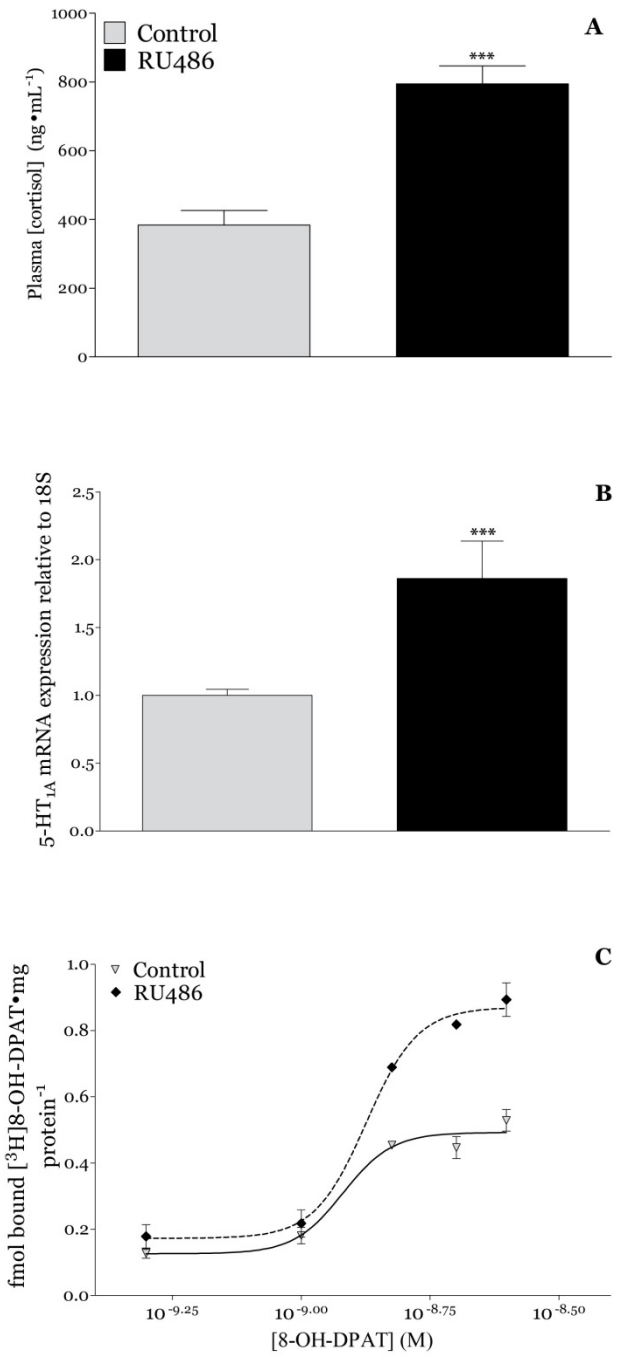

Figure 3.2. A. RU486-treated toadfish had twice the level of circulating cortisol concentrations compared to vehicle-injected controls ($p < 0.001$). B. The RU486 group expressed more 5-HT_{1A} mRNA transcript than control fish, implicating a role for GR in mediating changes in 5-HT_{1A} mRNA expression in the Gulf toadfish ($p < 0.001$). C. The 5-HT_{1A} mRNA expression pattern corresponded with 5-HT_{1A} receptor binding, with the RU486 treatment having a significantly higher B_{max} than the control treatment ($0.87 \pm 0.03 \times 10^{-15}$ mol·mg protein^{-1} and $0.49 \pm 0.023 \times 10^{-15}$ mol·mg protein^{-1}, respectively; $p < 0.05$). K_D did not differ significantly between treatments (RU486: $1.34 \pm 1.04 \times 10^{-9}$ mol·L^{-1}, control: $1.21 \pm 1.07 \times 10^{-9}$ mol·L^{-1}). Data are means ± s.e.m; N = 8; *** $p < 0.001$. For binding kinetics, data presented is from 8 combined brains per treatment group, s.e.m. represents N = 3 technical replicates.

physiological response to cortisol. Treatment with spironolactone caused a significant ($p < 0.05$), 50% decrease in circulating cortisol levels compared to the vehicle-injected controls (Fig. 3.3A). Despite the significant change in plasma cortisol levels, neither B_{max} (control: $2.41 \pm 0.28 \times 10^{-15}$ mol·mg protein^{-1}; spironolactone: $2.33 \pm 0.28 \times 10^{-15}$ mol·mg protein^{-1}) nor K_D (control: $3.31 \pm 1.15 \times 10^{-9}$ mol·L^{-1}; spironolactone: 3.49 ± 1.16

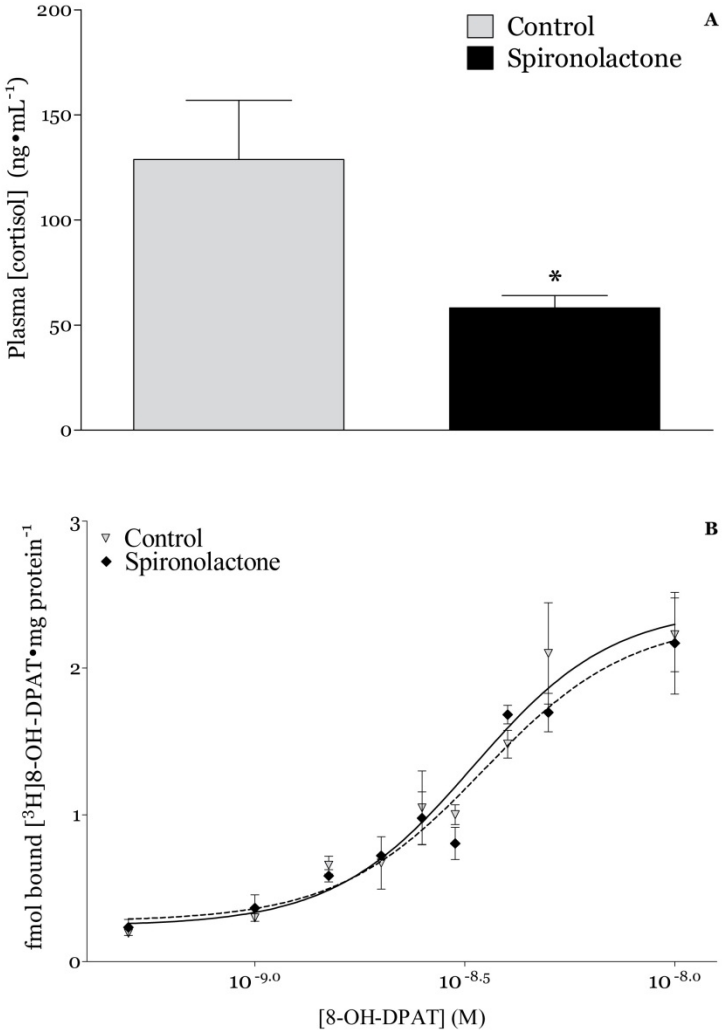

Figure 3.3. A. Spironolactone-treated toadfish had less than half the level of circulating cortisol concentrations compared to vehicle-injected controls ($p < 0.05$). B. No evident change in 5-HT$_{1A}$ receptor binding using [^{3}H]8-OH-DPAT based on either the B_{max} (control: $2.41 \pm 0.28 \times 10^{-15}$ mol·mg protein^{-1}; spironolactone: $2.33 \pm 0.28 \times 10^{-15}$ mol·mg protein^{-1}) or the K_D control: $3.31 \pm 1.15 \times 10^{-9}$ mol·L^{-1}; spironolactone: $3.49 \pm 1.16 \times 10^{-9}$ mol·L^{-1}). Data are means ± s.e.m; N = 8; * $p < 0.05$. For binding kinetics, data presented is from 8 combined brains per treatment group, s.e.m. represents N = 3 technical

replicates.

x 10^{-9} mol·L^{-1}) varied significantly between the two groups (Fig. 3.3B). While evaluating 5-HT_{1A} mRNA levels was attempted for spironolactone treatment, the housekeeping genes used (18S and elongation factor 1α) varied significantly between control and spironolactone-injected fish. While it is interesting that this treatment is affecting two of the more commonly used housekeeping genes, it made normalization impossible and so the analyses have been disregarded.

To determine whether RU486-treated fish had an enhanced functional response as a result of their higher 5-HT_{1A} receptor mRNA expression and B_{max}, the function of the 5-HT_{1A} receptor was tested *in vivo* on fish with indwelling caudal vein catheters using an 8-OH-DPAT challenge. Initial plasma cortisol levels in RU486-treated fish were five-fold higher than vehicle-injected control fish ($p < 0.001$; Fig. 3.4A). After the 8-OH-DPAT injection, the control group responded to 8-OH-DPAT with a significant, two-fold increase in circulating cortisol within 30 minutes ($p < 0.05$); surprisingly, there was no change in plasma cortisol in RU486-treated fish. To determine whether the elevated cortisol levels experienced by RU486-treated fish resulted in the inhibition of the functional response, we subjected cortisol-implanted fish to the 8-OH-DPAT challenge. Cortisol-implanted had initial plasma cortisol levels that were 10-fold higher than in control fish ($p < 0.01$; Fig. 3.4B). Only the control group responded to 8-OH-DPAT treatment with a significant 2-fold increase in circulating cortisol levels 30min post injection ($p < 0.05$); no changes were observed in cortisol-implanted fish. To determine whether MRs may be involved in mediating the functional downregulation, we subjected

spironolactone-treated fish to the 8-OH-DPAT challenge. In this case, initial plasma cortisol levels in spironolactone-treated fish were not significantly different than control

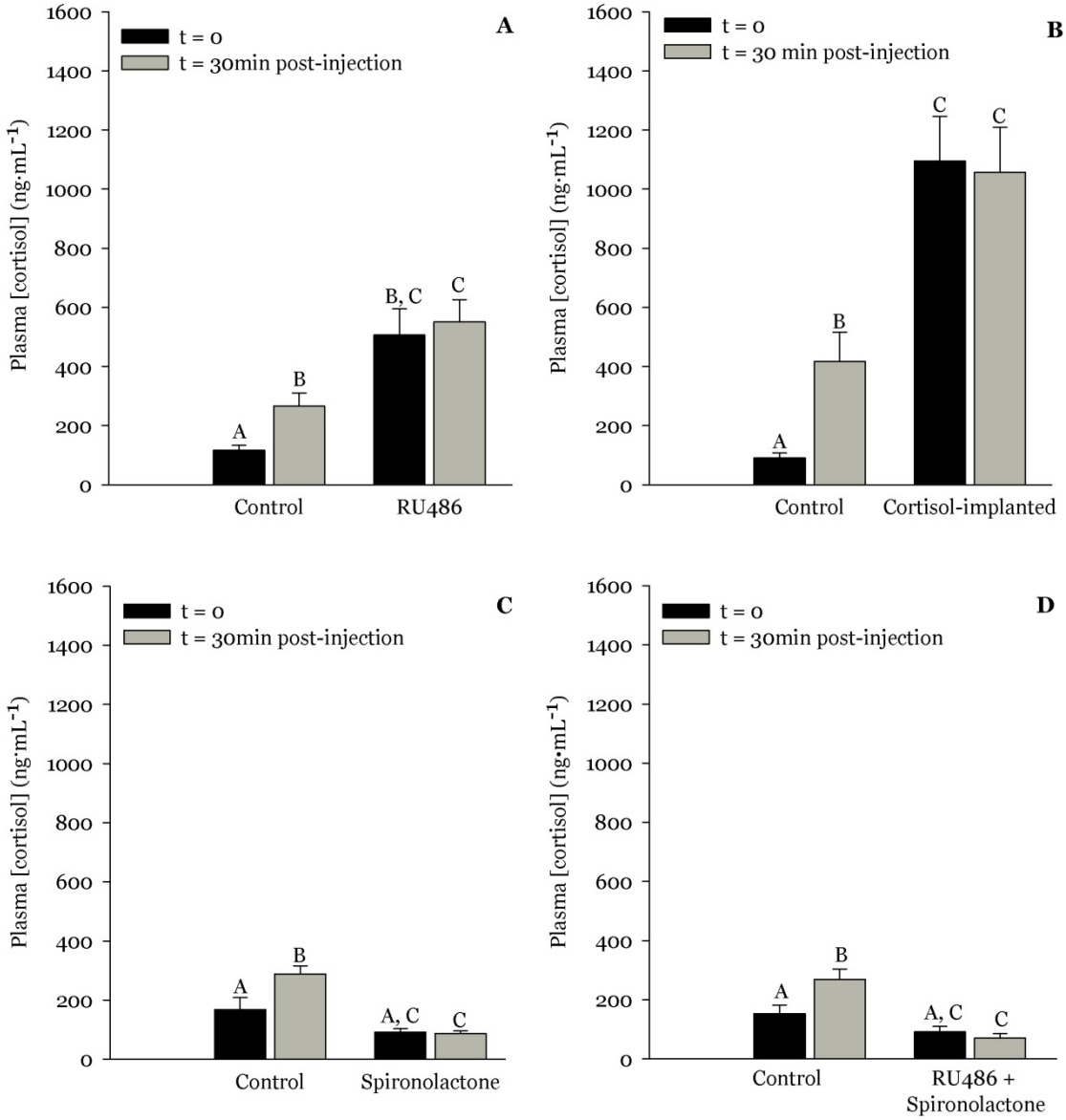

Figure 3.4. A. RU486-injected fish demonstrated significantly higher initial plasma cortisol levels compared to the vehicle-injected controls as a result of GR-blocking. Injection with 8-OH-DPAT failed to significantly increase circulating levels of cortisol in the RU486-treated group while the vehicle-injected controls experienced a 2-fold increase in their plasma cortisol levels. B. Cortisol-implanted fish showed significantly higher initial plasma cortisol levels compared to vehicle-implanted controls, but did not exhibit a response to 8-OH-DPAT. In contrast, the control group demonstrated a significant, 8-fold increase in cortisol levels ($p < 0.05$). C. Spironolactone-injected fish exhibited no initial difference in plasma cortisol levels when compared to control fish; however, there was no change in plasma cortisol of spironolactone-treated fish after injection with 8-OH-DPAT whereas the control group did experience a significant increase in plasma cortisol levels ($p < 0.05$). D. Co-treatment with both RU486 and spironolactone did not elicit a significant decrease in circulating levels of cortisol compared to vehicle-injected controls. As with the RU486-only and spironolactone-only, the controls were the only fish that

exhibited a reaction to injection with 8-OH-DPAT ($p < 0.05$). Data are means ± s.e.m; N = 8; different letters indicate a significant difference between treatments, $p < 0.05$.

fish. Similar to RU486-treated and cortisol-implanted fish, spironolactone-treated fish did not respond to 8-OH-DPAT; only the control group showed a significant 2.3-fold increase in plasma [cortisol] 30 minutes after 8-OH-DPAT injection ($p < 0.05$; Fig. 3.4C). To test whether GRs were compensating for the MRs when the MRs were blocked or vice versa, fish treated with both RU486 and spironolactone were subjected to the 8-OH-DPAT challenge. Initial plasma cortisol levels in the control group were not significantly different than the treated group ($p < 0.05$; Fig. 3.4D) and, again, only the control group experienced a significant increase in plasma cortisol post-8-OH-DPAT injection ($p < 0.05$).

Discussion

Many factors regulate the absolute level and stability of 5-HT_{1A} receptor mRNA, which, in turn, could lead to changes in the amount of receptor protein, and ultimately, the response in the whole animal. In mammals, circulating levels of corticosteroids have been shown to be one variable that regulates 5-HT_{1A} receptor mRNA expression in the brain (Chalmers and Watson, 1991; Chalmers et al., 1993; Zhong and Ciaranello, 1995; Wissink et al., 2000). In the present study, endogenous plasma cortisol levels doubled in response to crowding, supporting previous studies demonstrating that social interaction is a moderate stressor in teleost fish, including toadfish (Schreck, 1981; Wendelaar Bonga, 1997; McDonald et al., 2009; Pankhurst, 2010). We hypothesized that, like mammals, the elevation in cortisol would result in a decrease in 5-HT_{1A} mRNA expression in the brain and a corresponding decrease in 5-HT_{1A} receptor binding; and while there was no

difference in the mRNA expression of 5-HT_{1A} there was a significant decrease in maximal receptor binding (B_{max}) in crowded fish as compared to uncrowded fish. This suggests that the elevation in cortisol observed in response to the moderate, non-invasive crowding treatment may not be enough to mediate a change in the transcription of brain 5-HT_{1A} receptors but is high enough to mediate post-transcriptional or post-translational modifications in this receptor. Alternatively, as the sampling method used to determine mRNA expression evaluated long-term changes in 5-HT_{1A} mRNA levels (*i.e.*, changes after 1 week of crowding stress), there may have been an initial, transient change that was missed.

It cannot be completely ruled out that physiological changes in response to crowding treatment other than cortisol elevation (*e.g.*, an increase in 5-HT turnover) may be responsible for a downregulation in the 5-HT_{1A} receptor binding that is independent from changes in transcript levels (Kreiss and Lucki, 1997). However, 5-HT levels have not been shown to be elevated in crowded compared to uncrowded toadfish (AW Frere, MD McDonald, unpublished results). Furthermore, the role of cortisol alone in regulating changes in toadfish 5-HT_{1A} receptor mRNA expression and B_{max} is supported when comparing uncrowded, crowded and oil-injected fish; wherein differences in circulating cortisol levels between the groups resulted in graded changes in 5-HT_{1A} receptor physiology. As mentioned above, there was no measureable change in the 5-HT_{1A} mRNA expression in uncrowded fish compared to crowded fish, despite a four-fold elevation in cortisol levels. However, an inhibition in mRNA expression was measured in oil-injected controls (with cortisol levels that were twice as high as crowded fish) compared to the RU486-injected fish, further supporting the idea that a threshold stress

level may need to be met before prolonged transcriptional changes occur. In contrast to transcription, there was a clear relationship between plasma cortisol and 5-HT_{1A} receptor B_{max}, with a significant decrease in the B_{max} as cortisol levels increased in the control groups of each of these treatments (Fig. 3.5), suggesting that changes on the post-transcriptional level are more sensitive to cortisol than changes on the transcriptional

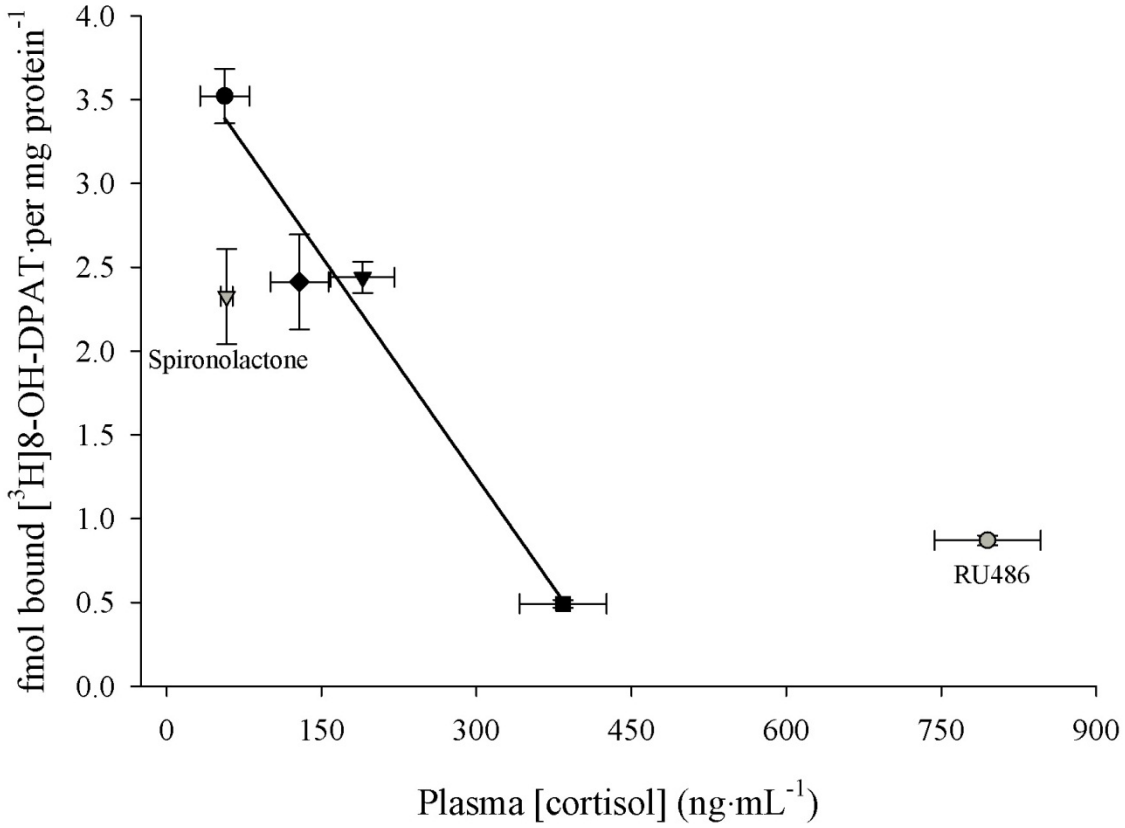

Figure 3.5. Plotting B_{max} (fmol bound [^{3}H]8-OH-DPAT·mg protein^{-1}) as a function of plasma cortisol concentration (ng·mL^{-1}) using uncrowded, crowded, and the peanut oil controls from the spironolactone- and RU486-treated groups resulted in a significant inverse correlation between plasma [cortisol] and 5-HT_{1A} protein level ($r^2 = 0.981$; $p = 0.02$). The B_{max} for the spironolactone- and RU486-treated fish are plotted separately (and were not included in the linear regression analysis) for comparison purposes.

level. Therefore, based on the results seen in this study, the response to subthreshold cortisol levels (such as those resulting from crowding) may be to alter existing protein levels, either by modifying G-protein coupling or internalization of the receptor, without a significant change in mRNA transcription. This type of protein modification may be

quicker and/or require less energy than effecting a prolonged change in mRNA transcription. While not as common as observing a change in transcription, corticosteroids have been shown to mediate post-transcriptional changes independent of transcriptional changes (Berg et al., 2004); for example, cortisol has been shown to decrease the amount of aquaporin 4 protein in sheep without affecting the mRNA expression of the aquaporin 4 gene (Ron et al., 2005). Furthermore, cortisol has been demonstrated to have post-translational effects on processing of gene products, affecting protein amount *in utero* (Fowden, 1995). To our knowledge, no studies, mammalian or otherwise, have observed a difference in 5-HT receptor protein binding that does not correspond to a significant change in mRNA transcription, making this the first observation of the potential effects of cortisol on post-transcriptional and/or post-translational processes affecting 5-HT receptors.

In teleosts, many long-term adaptive changes in response to cortisol are mediated by cytosolic GRs located in target tissues. Furthermore, both GRs and MRs are located throughout the HPI axis (Stolte et al., 2008; Greenwood et al., 2003; Vazzana et al., 2010; Arterbery et al., 2010) and have been shown to mediate canonical feedback whereby high cortisol levels inhibit the secretion of cortisol (Dallman et al., 2004; Fryer and Lederis, 1986). The GR antagonist RU486 has been shown to effectively block the physiological reaction to cortisol, interrupting the negative feedback of cortisol on the HPI axis (McDonald et al., 2004; Rodela et al., 2009; McDonald and Wood, 2004). This is evident in the present study, as RU486-treated fish showed a two-fold increase in cortisol levels compared to oil-treated controls. The inhibition of MRs by spironolactone, on the other hand, resulted in a decrease in toadfish plasma cortisol concentrations. This

result has been seen previously in fish (Sloman et al., 2001; Rodela et al., 2009; Schjolden et al., 2009); however, no explanation was provided and this finding actually contradicts some mammalian studies (Young et al., 1998 and Young et al., 2003), though the latter could be due to differences in treatment duration or organism. With spironolactone only, the decrease in plasma cortisol could be due to a negative feedback response that is initiated as cortisol is displaced from MRs and binds to GRs, as suggested by Berardelli et al. (2010); however, co-treatment with both spironolactone and RU486 still elicited a trend to decrease plasma cortisol suggesting that this may not be the case in toadfish.

While GRs have traditionally been implicated in the downregulation of 5-HT_{1A} mRNA in mammals in response to high levels of glucocorticoids due to pharmacological manipulation or a hyperfunctioning HPA axis (Burnet et al., 1992; Chalmers et al., 1993; Neumaier et al., 2000; Zhong and Ciaranello, 1995), MRs are thought to play a regulatory role at basal cortisol levels (Meijer and de Kloet, 1994; de Kloet et al., 2009). These roles are believed to be due, at least in part, to the higher affinity of the MR for cortisol compared to the GR, which results in the majority of MRs being occupied at basal cortisol levels (Cole et al., 2000). Supporting these ideas, an increase in 5-HT_{1A} mRNA transcription and a corresponding increase in 5-HT_{1A} receptor B_{max} were measured in RU486-treated fish relative to the oil-injected controls despite significantly higher circulating cortisol levels, suggesting that the downregulation in mRNA expression and B_{max} that should have been instigated by these very high cortisol levels (Fig. 3.5) are likely GR-mediated. Along these lines, an increase in 5-HT_{1A} receptor protein binding has been measured in GR-deficient (Hensler et al., 2010) mice and an increase in 5-HT_{1A}

receptor B_{max} has also been measured in adrenalectomized mice (Burnet et al., 1992; Martire et al., 1989; Zhong and Ciaranello, 1995) and mice with a hypofunctioning HPA axis (Burnet et al., 1992). However, it appears that treatment with RU486 does not completely abolish the effects of elevated cortisol, as indicated by the significant difference between the B_{max} of the RU486-treated and unstressed groups (Fig. 3.5). In contrast, toadfish treated with spironolactone showed no change in 5-HT_{1A} receptor binding kinetics compared to oil-treated controls, suggesting that, similar to mammals, these receptors may not play a role in the downregulation of 5-HT_{1A}, at least when cortisol levels are chronically elevated (de Kloet et al., 2009; Meijer and de Kloet, 1994). We might have expected that the significant decrease in plasma cortisol experienced by spironolactone treated fish would have resulted in an upregulation in 5-HT_{1A} receptor binding. Instead, spironolactone-treated fish fall outside the curve (Fig. 3.5), suggesting that despite cortisol levels similar to uncrowded fish, 5-HT_{1A} B_{max} primarily reflects the past stress associated with crowding and surgery, which likely had an initial inhibitory effect on mRNA transcription and 5-HT_{1A} receptor binding.

Injection with 8-OH-DPAT under control conditions elicits an increase in plasma cortisol concentrations in Gulf toadfish that peak 30 min post-injection (Medeiros et al., 2010). In the present study, the total amount of 5-HT_{1A} receptor protein, as suggested by the B_{max}, was higher in RU486 treated fish. Thus, we hypothesized that this would translate to more functional receptors and a greater cortisol elevation in response to 8-OH-DPAT compared to oil-treated fish. However, the increase in B_{max} did not translate to an increase in the functional response of the 5-HT_{1A} receptor, if, indeed, the functional response of 5-HT_{1A} is defined as an increase in circulating cortisol. In mammals, the

increase in cortisol measured in response to 8-OH-DPAT is actually believed to be due to the action of centrally located 5-HT_{1A} receptors (*i.e.*, those present in the hippocampus and pituitary) and the direct stimulation of either CRH (Calogero et al., 1990) or ACTH (Li et al., 1996; Van de Kar et al., 1998), respectively, which then stimulates the secretion of corticosteroids. In teleost fish, the stimulation of the 5-HT_{1A} receptor appears to result in a slight increase in ACTH that reflects a significant increase in cortisol secretion (Höglund et al., 2002; Winberg et al., 1997), but an investigation on the role of the 5-HT_{1A} receptor in the secretion of CRH has, to our knowledge, not been initiated. The direct activation of cortisol secretion via 5-HT_{1A} receptors in the interrenal tissue of fish likely plays a minor role, since there is minimal 5-HT_{1A} receptor mRNA expression in the kidney (Medeiros et al., 2010).

Given the conservation of 5-HT receptors throughout vertebrates, the most likely explanation may be that the functional response of the 5-HT_{1A} receptor, if instead measured as stimulation in CRH and/or ACTH secretion, is returned in toadfish upon treatment with RU486 like it is in rats (Akompong et al., 1993; de Kloet et al., 2009). That being the case, the apparent inhibition of 5-HT_{1A} receptor function would be due to elevations in cortisol affecting the ability of CRH and/or ACTH to elicit cortisol secretion from the interrenal cells. For example, a possible explanation for the apparent disconnect between protein level and cortisol secretion of the toadfish 5-HT_{1A} receptor in the present study could be at the level of the ACTH receptor. In coho salmon (*Oncorhynchus kisutch*) there is evidence for a decrease in ACTH receptor sensitivity in response to elevations in cortisol, which in the fish presents as a self-suppressive, non-GR mediated, non-genomic effect of cortisol acting via an ultra-short negative feedback loop at the

level of the interrenal cells (Bradford et al., 1992). Additionally, glucocorticoids are able to affect many different aspects of the stress axis, inhibiting many different secretagogues that would otherwise stimulate the release of corticosteroids (Osterlund and Spencer, 2011). Thus, in toadfish, perhaps the 5-HT_{1A} receptor is in fact functional in RU486- and/or spironolactone-treated fish, but some other aspect of the HPI axis that ultimately leads to cortisol secretion has been attenuated. That being said, there is evidence in mammals of a disconnect between 5-HT_{1A} transcript, protein levels and function. For example, inhibition of 5-HT cell discharge mediated by the 5-HT_{1A} receptor in the rat dorsal raphe nucleus is abolished in the presence of glucocorticoids independent of changes in transcription and protein binding (Laaris et al., 1995) and, in the rat spleen, the amount of the $G_{i\alpha}$ subunit, an important component of the G-protein secondary messenger system associated with 5-HT_{1A} receptors, is reduced by 60-70% after 24 h of dexamethasone treatment independent of 5-HT_{1A} transcript and protein levels (Akompong et al., 1993). In both cases this effect was clearly mediated by GRs, *i.e.*, the functional response returned upon treatment with RU486 (Akompong et al., 1993; de Kloet et al., 2009), and not MRs (de Kloet et al., 2009). Regardless of the direct action of 5-HT_{1A}, the results of the present study do suggest that an increase in 5-HT_{1A} receptor B_{max} does not necessarily translate to an overall increase in HPI axis stimulation; whether this is due to a direct interruption in the functional response of 5-HT_{1A} at the level of CRH or ACTH secretion in fish is currently being investigated.

In conclusion, the present study extends the findings of previous mammalian studies (Konings et al., 1995; Chalmers and Watson, 1991; Takao et al., 1997) to other vertebrates, showing that elevations in cortisol result in a GR-mediated downregulation

of brain 5-HT_{1A} mRNA expression that leads to changes in the amount of receptor protein in the Gulf toadfish. Interpreting the functional response of 5-HT_{1A} in the face of elevated circulating cortisol and/or GR and/or MR inhibition is slightly more difficult and complicated by the fact that the 5-HT_{1A} receptor likely plays a direct role at the level of the hypothalamus and pituitary and the whole HPI axis response (*i.e.*, the secretion of cortisol) involves many other potential regulatory factors. The fact that RU486, spironolactone and high circulating cortisol all have the ability to inhibit the whole HPI axis response to injection with 8-OH-DPAT suggests the necessity of functional GRs, MRs and low circulating cortisol levels to mediate cortisol secretion via the 5-HT_{1A} receptor, perhaps further supporting the sensitivity of this receptor to negative feedback.

CHAPTER 4
CROWDING STRESS ATTENUATES THE SEROTONIN SUBTYPE 1A RECEPTOR-MEDIATED SECRETION OF CORTICOTROPIN RELEASING FACTOR AND ADRENOCORTICOPIN HORMONE IN THE GULF TOADFISH

Background & significance

In mammals, it is well established that the monoamine neurotransmitter, serotonin (5-HT; 5-hydroxytryptamine) stimulates the release of cortisol from the adrenal gland (Chaouloff, 1993; Dinan, 1996). Consistent with conserved brain organization, including monoaminergic pathways, among vertebrates (Parent, 1981; Parent et al., 1984; Nieuwenhuys et al., 1998), 8-hydroxy- 2-(di-n- propylamino) tetraline (8-OH-DPAT), an agonist of mammalian 5-HT_{1A} receptors, elevates plasma corticosteroid levels in both mammals and multiple species of teleost fish (Bovetto et al., 1996; Fuller, 1990; Medeiros et al., 2010; Van de Kar et al., 1989; Winberg and Nilsson, 1996; Winberg and Nilsson, 1993; Zifa and Fillion, 1992).

The Gulf toadfish 5-HT_{1A} receptor is nearly 70% homologous to the human gene (Medeiros et al., 2010), with other species of teleost also showing the same high level of similarity to the human homologue (Wang and Tsai, 2006; Yamaguchi and Brenner, 1997). In regards to the hypothalamic-pituitary-interrenal (HPI) axis, which is responsible for cortisol secretion in teleost fish, the expression of toadfish 5-HT_{1A} receptor mRNA is mostly found in the pituitary and the midbrain area, with very low or undetectable levels of transcript found in kidneys (which contains the interrenal cells) (Medeiros et al., 2010). Pharmacologically, the characteristics of the toadfish 5-HT_{1A} receptor are very similar to those measured in both mammalian (Barnes and Sharp, 1999; Peroutka and Howell, 1997) and other teleost (Winberg and Nilsson, 1996) studies,

exhibiting similar binding affinities (K_D) for both 5-HT and 8-OH-DPAT (Medeiros et al., 2010). Furthermore, similar to its action in mammals, 8-OH-DPAT has been shown to cause an increase in plasma cortisol levels within 30 minutes in the Gulf toadfish (Medeiros et al., 2010) as well as the rainbow trout (Winberg et al., 1997) at concentrations similar to the binding affinities observed in mammals by Peroutka and Howell (1997).

Thus, 5-HT, mediated by the 5-HT_{1A} receptor, is playing a role in regulating the toadfish stress response; however, it is not known at what levels of the HPI axis the 5-HT_{1A} receptor may be responsible for ultimately stimulating the release of cortisol. Based on the low level of 5-HT_{1A} receptor mRNA transcript present in the kidney of the Gulf toadfish, it is not likely that the receptor is directly responsible for the secretion of cortisol from the interrenal cells (Medeiros et al., 2010). Supporting the idea that the pituitary may be involved in mediating the activation of the HPI axis is a study by Höglund et al. (2002) in which there was a tendency for plasma [ACTH] concentrations to increase following intraperitoneal (IP) injection of 8-OH-DPAT via a permanent IP catheter injection, an increase that mirrored a dose-dependent increase in plasma cortisol levels. The elevation in circulating ACTH levels in that study was not statistically significant; however, this might have been due to the fact that the sampling period was 1h post-injection and transient elevations in ACTH could have been missed as ACTH is readily broken down by proteases. To our knowledge, the role of 5-HT_{1A} receptors mediating an increase in CRF in the POA of teleosts has never been investigated. One of the aims of the present study is to discover whether the 5-HT_{1A} receptor population in the POA and/or the pituitary, or even neither) is responsible for mediating such an increase in

circulating levels of cortisol. Based on our mRNA expression analysis (Medeiros et al., 2010), we hypothesize that separate 5-HT_{1A} receptor populations in the POA and pituitary are responsible for stimulating the independent release of CRH and ACTH, respectively, leading to an increase in circulating cortisol levels.

In mammals, it is well established that corticosteroids, such as cortisol, downregulate transcription and binding affinity of the 5-HT_{1A} receptor (Beer et al., 1990; Blier and de Montigny, 1987; Fanelli and McMonagle-Strucko, 1992; Gobbi et al., 1991; Kennett et al., 1987; Schechter et al., 1990) and there is evidence for a similar trend in the Gulf toadfish (Medeiros and McDonald, 2012). Medeiros and McDonald (2012) showed that crowding stress appeared to lower the maximum binding (B_{max}) of the toadfish receptor; an effect that could be reversed upon administration of RU486. Furthermore, 5-HT_{1A} function in the face of elevated cortisol also appeared to be impaired; however, the role of GRs in mediating this functional response was confounding (Medeiros and McDonald, 2012). Thus, the second aim of this study is to investigate how crowding is affecting CRH and ACTH secretion at the level of the POA and pituitary, respectively. We hypothesize that the function of centrally activated 5-HT_{1A} receptors is being reduced or abolished as a result of chronically elevated plasma cortisol levels and that this response is GR-mediated.

Materials and methods

Experimental animals

Gulf toadfish (*Opsanus beta*) were captured by commercial shrimpers using roller trawls in Biscayne Bay, Florida in the fall and winter of 2011, after which they were then

immediately transferred to the laboratory where they were held for up to one month. Upon arrival in the lab, fish were placed in fresh water for 5 minutes and then treated with a dose of malachite green (final concentration 0.05 mg l^{-1}) in formalin (15 mg l^{-1}) (AquaVet) to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993). The fish were kept in 50-liter glass aquaria with flowing, aerated seawater at a temperature of 20-22ºC and were fed weekly with squid.

Experimental Treatments

Series 1: Lab uncrowded fish

Lab uncrowded (approximately 20 g fish L^{-1}) toadfish (N = 32) were used to determine if intravenous injection of the 5-HT_{1A} agonist, 8-OH-DPAT, resulted in an increase in CRF precursor mRNA, a proxy for CRF levels (Alderman and Bernier, 2007; Backstrom et al., 2011; Bernier, 2006; Bernier et al., 2008; Bernier et al., 1999; Chen and Fernald, 2008; Craig et al., 2005; Fuzzen et al., 2010), in the preoptic area (POA) of the brain (mean mass 0.064 ± 0.003 kg, N = 16) and/or an increase in plasma ACTH (mean mass 0.074 ± 0.004 kg, N = 16). To do this, indwelling caudal vein catheters were inserted as described by McDonald et al. (2000). Briefly, the caudal vertebrae were exposed by a 1.5–2.0 cm lateral incision between the epaxial and hypaxial muscle masses. The haemal arch was cannulated with PE50 tubing (Clay-Adams) filled with heparanized saline (50 i.u mL^{-1} sodium heparin; Sigma-Aldrich). A heat-flared PE160 (Clay-Adams) sleeve was then glued in place with cyanoacrylate tissue cement and sutured at the site of exit to secure the catheter. To prevent infection, the wound was then treated with oxytetracycline powder and sutured securely with 3-0 silk. Following surgery, fish were

allowed to recover for a full day before the 8-OH-DPAT challenge (see details below in *Experimental Protocols*).

Series 2: Lab uncrowded fish with antalarmin treatment

To elucidate if the Gulf toadfish 5-HT_{1A} receptor, which is hypothesized to stimulate the synthesis and release of adrenocorticotropic hormone (ACTH) from the mammalian pituitary, is involved in directly stimulating the release of ACTH from the Gulf toadfish pituitary, lab uncrowded fish were treated with the mammalian CRF receptor antagonist, antalarmin (Sigma-Aldrich). Intraperitoneal (IP) catheters (PE160) were implanted as described by McDonald and Walsh (2004). The IP catheter, pre-filled with Tween 80 (Sigma-Aldrich), as described by Lastein et al. (2008), was inserted through a small ventral incision and threaded approximately 4 cm inside the body cavity. Immediately following insertion of the IP catheter, caudal catheters were surgically inserted into fish as described above. Following surgery, fish were allowed to recover for a full day. To prove that antalarmin effectively blocks the toadfish CRF receptor, a pilot experiment was undertaken in which a group of lab-acclimated Gulf toadfish (mean mass 0.093 ± 0.009 kg, N = 6) received an antalarmin injection (2 mg antalarmin·0.5 ml Tween 80^{-1}·100 g $fish^{-1}$) while another group (mean mass 0.068 ± 0.011 kg, N = 6) received an injection of the vehicle alone (Tween 80; 0.5 ml Tween 80^{-1}·100 g $fish^{-1}$) 1 h prior to the administration of CRF (Tocris Biosciences) at a concentration of 0.64 μg·0.1 ml $saline^{-1}$·100 g $fish^{-1}$. Once the effectiveness of antalarmin in inhibiting the toadfish CRF receptor was verified (*i.e.*, there was no increase in ACTH in response to CRF injection in antalarmin-treated fish), the same dose of antalarmin (mean mass 0.064 ± 0.004 kg, N

= 6) or vehicle alone (mean mass 0.067 ± 0.005 kg, N = 6) was given to fish 1 h prior to the 8-OH-DPAT challenge (described below).

Series 3: Lab crowded fish with or without RU486 treatment

To determine if crowding, which is known to decrease whole-brain 5-HT_{1A} receptor binding, results in a downregulation in 5-HT_{1A} receptor-mediated CRF and ACTH secretion, and if the glucocorticoid receptor (GR) is involved in mediating that response, fish were subjected to 1 week of crowding followed by 48h of treatment either with peanut oil alone or with the GR antagonist, RU486 (mifepristone; Sigma-Aldrich), in peanut oil. A total of *N* = 40 fish (*N* = 20 fish for CRF and an N = 20 for ACTH assessment) were subjected to one week of crowding (approximately 70 g fish L^{-1}) in one of two 10 L tubs in the laboratory. Out of those 2 x 20 fish, the smallest 16 toadfish (to be consistent with social status) from each tank were anesthetized (MS 222; 1 $g \cdot L^{-1}$) prior to being surgically implanted with an IP catheter filled with peanut oil, as described above in *Series 2*. Immediately following insertion of the IP catheter, caudal vein catheters were surgically placed into fish, as described in *Series 1*. After surgery, the fish were placed in individual 2 L tubs and left to recover undisturbed for 24 h. After the recovery period, fish were injected intraperitoneally with either peanut oil alone (0.2 mL peanut oil·100 g $fish^{-1}$ followed by 0.3 mL of peanut oil; mean mass 0.075 ± 0.004 kg, *N* = 15) or 10 mg RU486·0.2 mL peanut $oil^{-1} \cdot 100$ g $fish^{-1}$ followed by 0.3 ml of peanut oil (mean mass 0.075 ± 0.004 kg, *N* = 16). The doses used were based on results from preliminary experiments as well as previous studies on fish (McDonald et al., 2004;

Rodela et al., 2009; Medeiros and McDonald, 2012). Injections were repeated every 12h for 48h of treatment, after which fish underwent the 8-OH-DPAT challenge (see below).

Experimental protocols

8-OH-DPAT challenge. The function of the 5-HT_{1A} receptor *in vivo* was assessed with an 8-OH-DPAT challenge as described by Medeiros et al. (2010). Initial 200 µL blood samples were drawn via the caudal vein catheter immediately before intravenous injection with 8-OH-DPAT at a dose of 1.63 mg·0.1 ml $saline^{-1}$·100 g $fish^{-1}$ or, in the case of the control group for *Series 1*, saline (0.1 ml saline·100 g $fish^{-1}$). This dose has been established by Medeiros et al. (2010) to cause a significant increase in plasma cortisol levels within 30 min in Gulf toadfish. To determine if levels of CRF changed in the POA in response to 8-OH-DPAT injection, 3 minutes post-injection saline or 8-OH-DPAT injection, fish were anesthetized with a lethal dose of MS 222 (3 g·L^{-1}) and the brain was quickly, but carefully removed and placed on a piece of plexiglass to prevent the organ from drying. Stressing efficiency and consistency, the preoptic area and most of the hypothalamic region (referred to as the POA throughout this manuscript; Fig. 4.1) was carefully dissected from the other brain tissue, frozen immediately in liquid N_2 and stored at -80ºC until RNA extractions were performed. A second subset of fish was used for the quantification of ACTH release. These fish were left for 15 minutes post- saline or 8-OH-DPAT injection, at which time a final (200µL) blood sample was drawn via the caudal catheter. Blood samples were centrifuged with a drop of 15% EDTA (10 µL) at 16,000 *g* for 5 min and the plasma decanted. Plasma samples were frozen immediately in liquid N_2 and stored at -80ºC for later analyses of cortisol or ACTH levels.

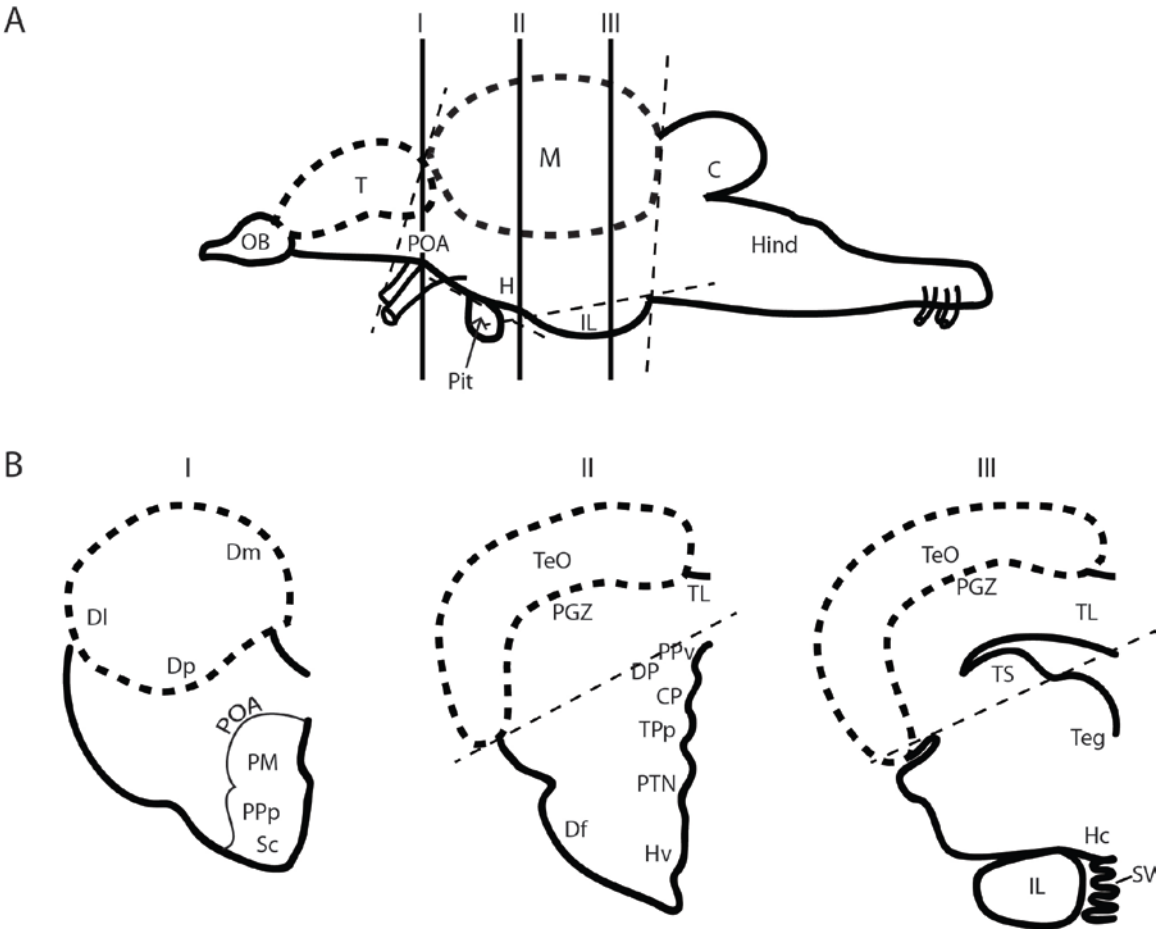

Figure 4.1. A. Schematic mid-sagittal diagram depicting the way in which the brain was sectioned, with transverse lines corresponding to transverse hemisections (see panel B). B. Transverse hemisection diagrams indicating finer scale sectioning lines as well as approximate anatomical regions for reference (modified from Alderman and Bernier, 2007; Forlano et al., 2010). Sectioning was intended to separate the preoptic area (POA) and hypothalamic region from the remainder of the brain. Dashed lines indicate where an incision was made.

Cloning of the Gulf toadfish CRF precursor gene. A fragment of the CRF precursor gene was cloned from toadfish brain cDNA using degenerate primers (Table 4.1) designed using alignments of other teleost fish. Total RNA was isolated from brain following the protocol provided with the Trizol reagent (Invitrogen). Total RNA was subsequently treated with DNAse I to remove potential residual genomic DNA according to the protocol provided with the TurboDNA-free kit (Ambion). cDNA was synthesized using

Oligo(dT) primers from 1 μg of DNase I-treated total RNA according to the protocol provided with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR reactions were performed using GoTaq DNA polymerase (Promega) and the following cycling conditions: 94°C for 30 s, a temperature gradient of 50-70°C for 1 min, and 72°C for 1 min for 40 cycles. Products were gel-purified, cloned using the TOPO TA vector (Invitrogen) and sequenced (Genewiz, Inc; South Plainfield, NJ).

Quantitative (real-time) PCR (qPCR) analysis of CRF precursor mRNA. Total RNA was isolated, DNAse I treated and cDNA was synthesized as described above. 'No reverse transcriptase' controls were routinely performed as an additional quality check. A ten-fold dilution of the cDNA product was made up using double-deionized RNA- and DNA-free water (Sigma Aldrich). Primer sequences for the normalizing gene, EF1α, were obtained from Grosell et al. (2009) (Table 4.1). Primers for the CRF precursor gene (Table 4.1) were designed based on the sequence of the cloned and sequenced toadfish CRF fragment and the products were gel-purified and then sequenced (Genewiz, Inc; South Plainfield, NJ) to confirm that the primers were indeed amplifying toadfish CRF. Quantitative PCR was performed using an Mx3005P Multiple Quantitative PCR system

Table 4.1. Primers used for qPCR and cloning of the Gulf toadfish CRF precursor hormone

Primer	Sequence (5'→3')	Product Size (bp)
CRF-F*	TNG GDG AGG ART AYT WCA TCC G	~222
CRF-R*	AAS GTV AGR TCY ARD GAD ATC GG	
qPCR CRF-F	TTC ATC CGA CTG GGC AAC GG	147
qPCR CRF-R	ATC GGC GGG TCG TCG GAC CTC CTT	
EF1α-F	AGG TCA TCA TCC TGA ACC AC	143
EF1α-R	GTT GTC CTC AAG CTT CTT GC	

***Degenerate primer sequences used for initial cloning of toadfish CRF precursor fragment, designed from partially conserved regions of other teleost CRF precursor aligned sequences**
Abbreviations: F, forward primer; R, reverse primer

(Stratagene) with Power SYBR Green qPCR Master Mix (Life Technologies) as the reporter dye. Cycling parameters were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. At least 6 separate biological replicates, representing cDNA isolated from at least 6 individual fish, were used for each treatment. Fold-changes between treatments were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The standard curves of the gene of interest and normalizers genes in this study gave PCR efficiencies of 100.4% (CRF; R^2=0.97) and ≥95% (EF1α; R^2=1.0; Grosell et al., 2009). To confirm that there was no contamination or primer dimer formation contributing to the fluorescence measured, 'no reverse transcriptase' and 'no template controls' were performed.

Analytical Techniques. Plasma cortisol and ACTH were quantified using the respective MP Biomedical ^{125}I RIA kit, with the cortisol standards diluted by half so that protein concentrations were within the range measured in toadfish. ACTH standards were used as provided.

Statistics. Unless otherwise noted, analyses were conducted using SigmaStat and/or SigmaPlot (SPSS, Inc.). Data are given as means ± 1 s.e.m., and N = the number of fish in each assay. To determine differences within treatments, a Repeated Measures ANOVA was performed. To determine differences amongst treatments, an ANOVA was performed. Both were followed by a Bonferroni post-hoc test when a significant difference was found ($p<0.05$).

Results

A 222 nucleotide (74 amino acid) fragment of the Gulf toadfish CRF precursor gene, which comprises 44% of the total gene, was successfully cloned and sequenced. On the amino acid level, the cloned fragment was found to share between 66 and 86% similarity with other species of teleost fish (Fig. 4.2), with the highest similarity shared with tilapia (*Tilapia mossambica*).

To elucidate the role the 5-HT_{1A} receptor plays in the secretion of ACTH and CRF at the level of the hypothalamus and pituitary, respectively, the effect of intravenous injection with the 5-HT_{1A} receptor agonist 8-OH-DPAT on CRF precursor mRNA levels and plasma ACTH concentrations was evaluated in lab uncrowded Gulf toadfish of *Series 1*. Pre-injection cortisol values were not significantly different between control and 8-OH-DPAT-injected fish (data not shown). However, 15 minutes post-injection there was

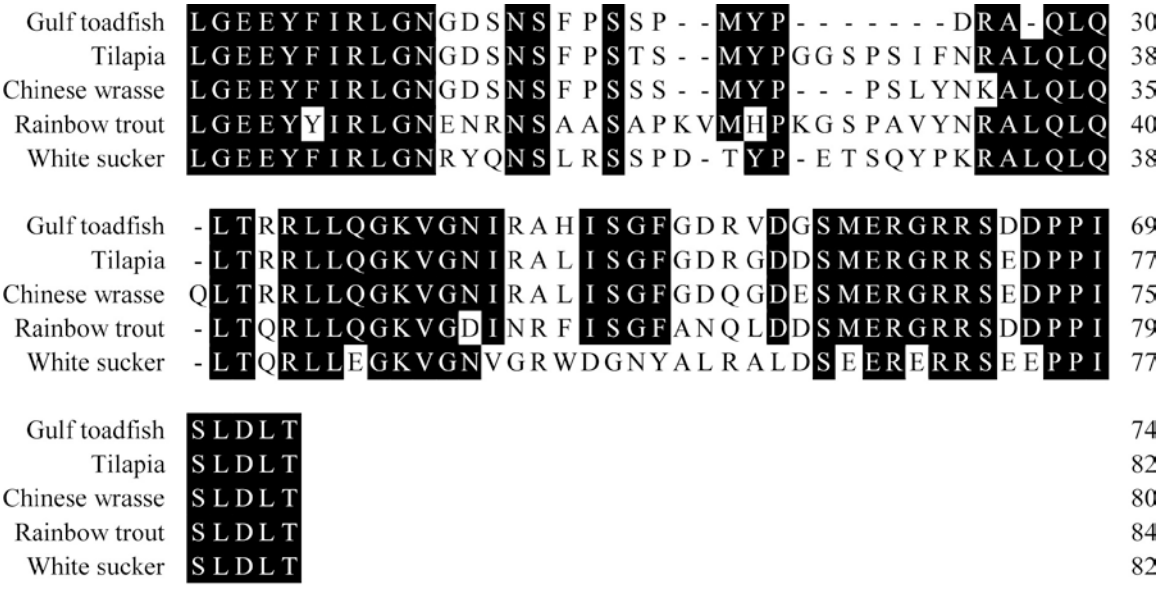

Figure 4.2. CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) amino acid alignment of translated nucleotide CRF sequences from Gulf toadfish (*Opsanus beta*), submission ID **1523266**; Tilapia (*Oreochromis mossambicus*), AY219038; Chinese wrasse (*Halichoeres tenuispinis*), DQ073097; Rainbow trout (*Oncorhynchus mykiss*), AF296672; and White sucker (*Catostomus commersonii*), J04116. Shaded amino acids indicate that at least 80% of the sequences possess that particular amino acid. Alignment created using Multiple Align Show (http://www.bioinformatics.org/SMS/multi_align.html).

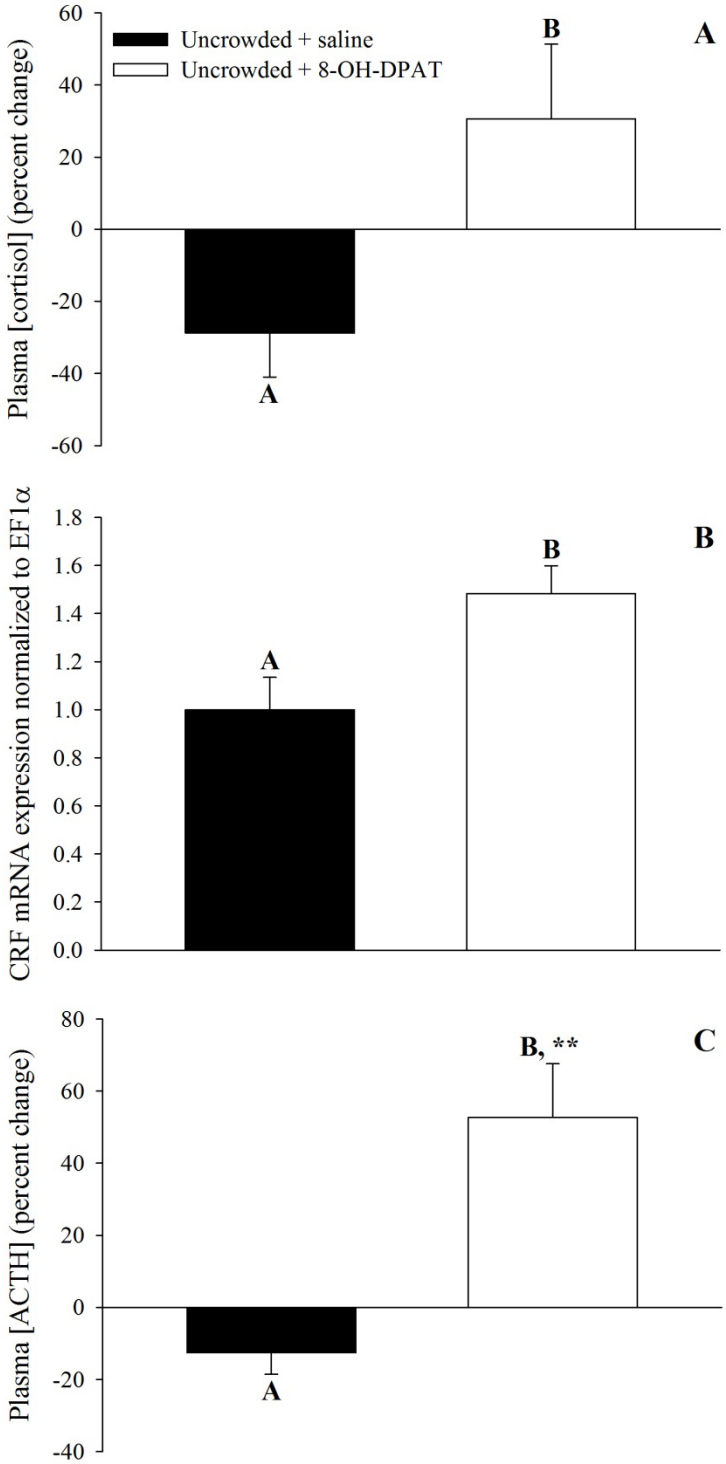

Figure 4.3. A. The percent change of plasma [cortisol] in the Gulf toadfish (*Opsanus beta*) 15 minutes post-injection with either saline or 8-OH-DPAT. B. Relative quantity of CRF precursor mRNA expression present in the POA 3 minutes after injection with either saline or 8-OH-DPAT; transcript levels are normalized to the housekeeping gene EF1α and quantities relative to the saline-injected control. C. The percent change of plasma [ACTH] in the Gulf toadfish (*Opsanus beta*) 15 minutes post-injection with either saline or 8-OH-DPAT. Values are means ± s.e.m.; N = 8; ** $P < 0.01$ significantly different relative to initial value; $P < 0.05$ different letters represent a significant difference between treatments.

a significant 30.6% increase in plasma cortisol in the 8-OH-DPAT-injected fish compared to a 28.8% decrease in the saline-injected controls ($P < 0.05$; Fig. 4.3A). CRF precursor mRNA expression levels in the 8-OH-DPAT-injected fish were elevated by 1.4-fold compared to the saline-injected control ($P < 0.05$; Fig. 4.3B) 3 minutes post-injection. Furthermore, plasma [ACTH] increased by approximately 50% ($P < 0.01$) a change that was 1.8-fold greater than saline-injected fish ($P < 0.05$; Fig. 4.3C).

To test whether the mammalian-based drug, antalarmin, a CRF receptor antagonist, effectively blocked the toadfish CRF receptor in the pituitary, lab uncrowded toadfish treated with the vehicle alone (Tween 80) or vehicle + antalarmin were injected with CRF and plasma ACTH concentrations were measured. Vehicle-treated fish showed a significant 38.1% increase in plasma ACTH concentrations in response to CRF injection ($P < 0.01$) whereas antalarmin-treated fish did not show a significant change (Fig. 4.4A). Having provided evidence that antalarmin effectively blocks CRF-mediated increases in plasma ACTH concentrations in the Gulf toadfish, 8-OH-DPAT was administered to a different set of vehicle and antalarmin-treated fish to separate out the effects of 5-HT_{1A} receptor-mediated CRF secretion on ACTH secretion from the potential direct effect of 5-HT_{1A} receptors known to be present in the pituitary. Both vehicle-treated and antalarmin-treated fish experienced a significant 60% increase in plasma ACTH concentration in response to 8-OH-DPAT injection ($P < 0.001$ and $P < 0.01$, respectively) that were not significantly different from each other (Fig. 4.4B).

To determine if crowding, which is known to decrease whole-brain 5-HT_{1A} receptor binding, is affecting 5-HT_{1A} receptor-mediated CRF and ACTH secretion, and if the glucocorticoid receptor (GR) is involved in mediating that response, fish were

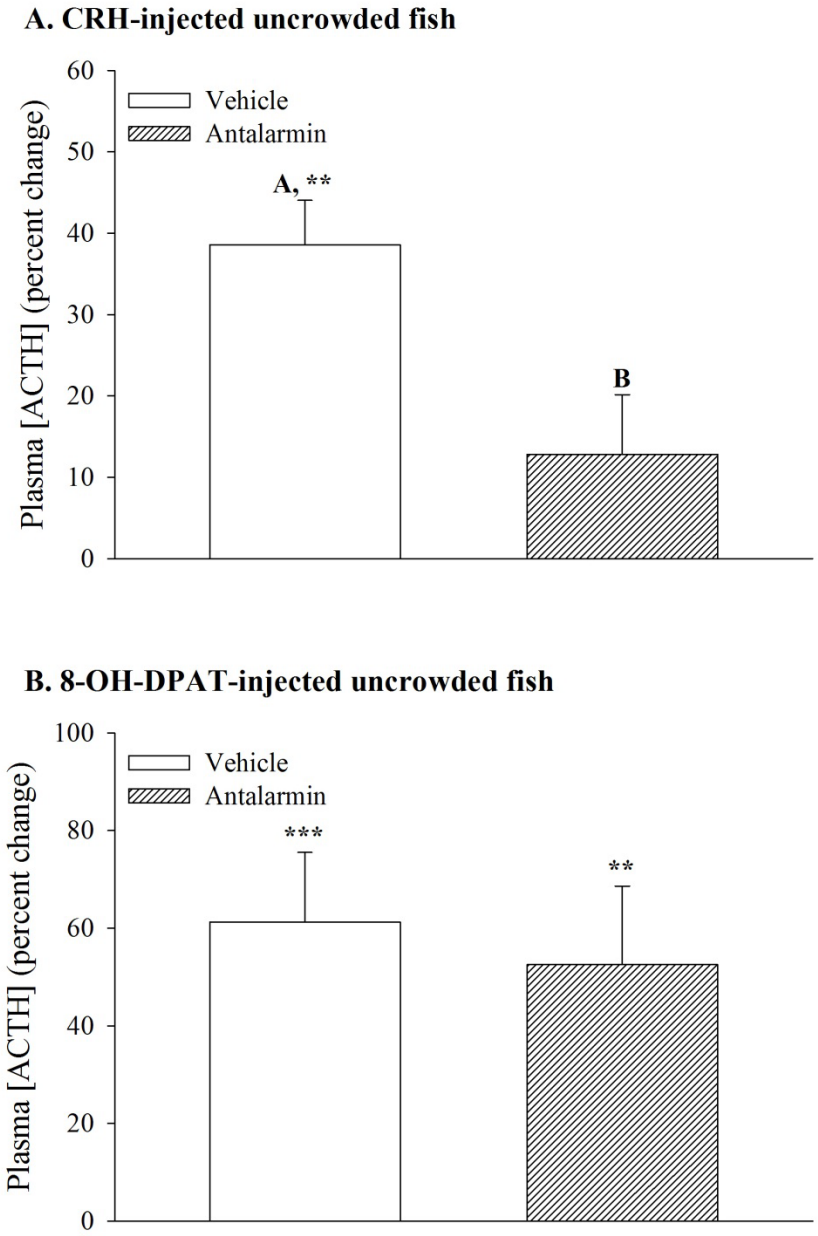

Figure 4.4. A. The percent change of plasma [ACTH] in the Gulf toadfish (*Opsanus beta*) 15 minutes post-injection with CRF after being treated with either vehicle alone (Tween 80) or vehicle with antalarmin 1 h prior to injection with CRF. B. The percent change of plasma [ACTH] in the Gulf toadfish (*Opsanus beta*) 15 minutes post-injection with 8-OH-DPAT after being treated with either vehicle alone (Tween 80) or vehicle with antalarmin 1 h prior to injection with 8-OH-DPAT. Values are means ± s.e.m.; N = 6;** $P < 0.01$, *** $P < 0.001$ significantly different relative to initial value; $P < 0.05$ different letters represent a significant difference between treatments.

subjected to 1 week of crowding followed by 48h of treatment either with peanut oil (crowded vehicle-treated) or with the GR antagonist, RU486, in peanut oil (crowded RU486-treated). These fish were then subjected to the 8-OH-DPAT challenge.

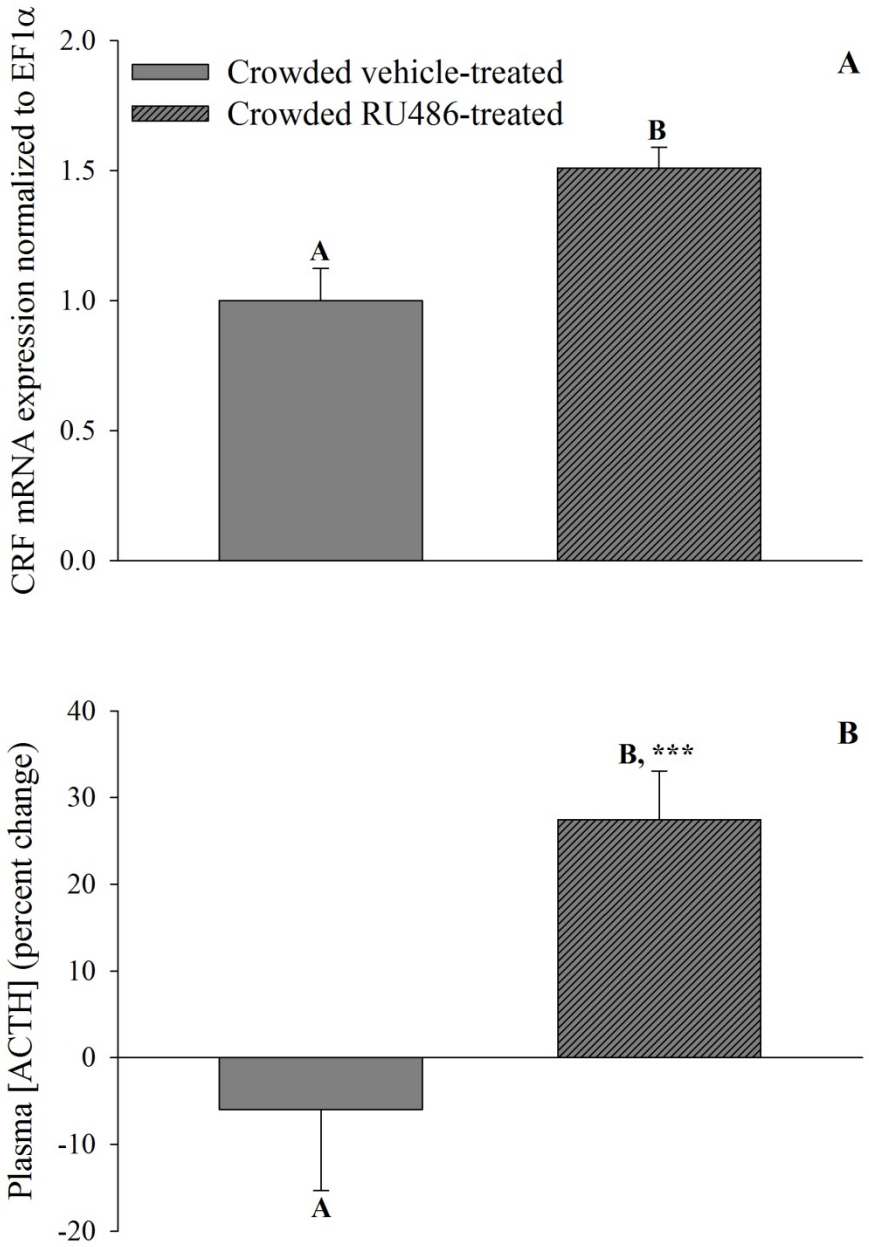

Figure 4.5. A. Relative quantity of CRF precursor mRNA expression present in the POA of Gulf toadfish (*Opsanus beta*) 3 minutes after injection with 8-OH-DPAT after 1 week of crowding followed by 48 h of treatment with either vehicle (peanut oil) or vehicle + RU486. Transcript levels are normalized to the housekeeping gene EF1α and quantities relative to the vehicle-treated group. B. The percent change of plasma [ACTH] in the Gulf toadfish (*Opsanus beta*) 15 minutes post-injection with 8-OH-DPAT after 1 week of crowding followed by 48 h of treatment with either vehicle (peanut oil) or vehicle + RU486. Values are means ± s.e.m., N = 8, *** $P < 0.001$ relative to initial value, different letters represent a significant difference between treatments ($P < 0.05$).

Following injection with 8-OH-DPAT, CRF precursor mRNA expression levels were elevated in the crowded RU486-injected fish by approximately 51% ($P < 0.01$) compared to the crowded vehicle-treated controls (Fig. 4.5A). Interestingly, crowded + vehicle-

treated fish did not experience a significant increase in circulating levels of ACTH in response to injection with 8-OH-DPAT, whereas the plasma ACTH concentrations of crowded RU486-injected fish increased by approximately 30% ($P < 0.001$; Fig. 4.5B) resulting in RU486-treated fish having plasma ACTH levels that were 1.8-times higher than the vehicle-treated fish ($P < 0.01$).

The CRF precursor mRNA expression responses between lab-uncrowded, crowded + vehicle- and crowded +RU486-treated 8-OH-DPAT-injected fish were compared to lab-uncrowded fish that were injected with saline (controls, signified by the dotted line) demonstrating that 8-OH-DPAT-injection resulted in a 1.45-fold higher CRF precursor transcript levels in uncrowded fish compared to saline-injected controls ($P < 0.001$; Fig. 4.6A). This was in contrast to the responsiveness of crowded + vehicle-treated fish, which showed no response to 8-OH-DPAT compared to saline-injected controls. Upon treatment with RU486, the response to 8-OH-DPAT injection returned, resulting in a 1.75-fold increase in CRF precursor levels compared to saline-injected controls ($P < 0.001$). The CRF precursor response of the uncrowded and crowded + RU486-treated fish to 8-OH-DPAT injection did not differ but were both significantly higher than crowded + vehicle-treated fish. A similar pattern was evident in plasma ACTH concentrations in response to 8-OH-DPAT injection in these same groups of fish (Fig. 4.6B).

Discussion

While 5-HT_{1A} receptor-mediated increases in CRF and ACTH have been measured in mammals (Calogero et al., 1989; Kageyama et al., 1998; Martinez and

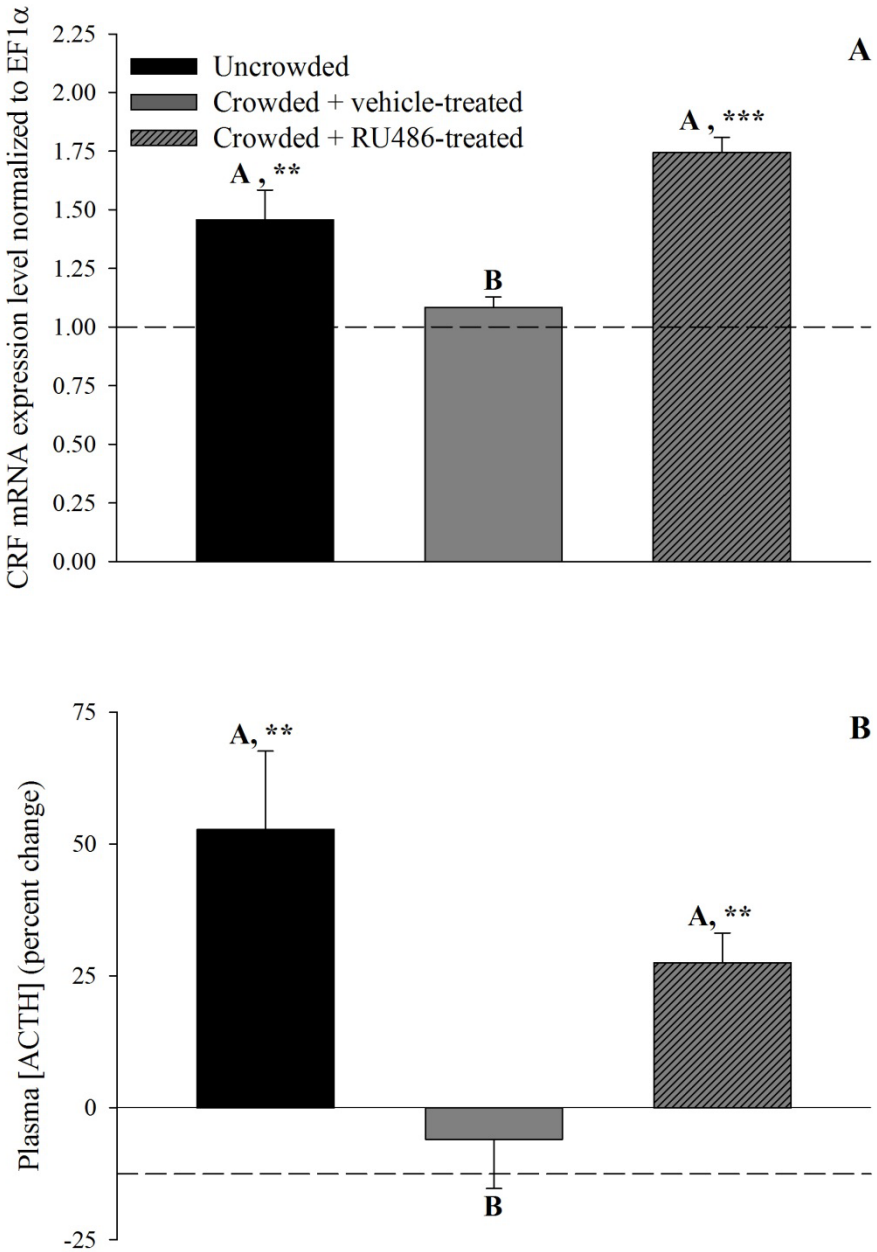

Figure 4.6. A. Relative quantity of CRF precursor mRNA expression present in the POA of Gulf toadfish (*Opsanus beta*) 3 minutes after injection with 8-OH-DPAT in lab-uncrowded fish or after 1 week of crowding followed by 48 h of treatment with either vehicle (peanut oil) or vehicle plus RU486. Transcript levels are normalized to the housekeeping gene EF1α and quantities relative to saline-injected lab-uncrowded fish from *Series 1* (represented by the dashed line). B. The percent change of plasma [ACTH] in the Gulf toadfish (*Opsanus beta*) 15 minutes post-injection with 16.25 mg·kg^{-1} 8-OH-DPAT in the same groups of fish. Values are means ± s.e.m., N = 8, ** $P < 0.01$, *** $P < 0.001$ relative to saline-injected lab-uncrowded fish from *Series 1* (represented by the dashed line), different letters represent a significant difference between treatments ($P < 0.05$).

Bueno, 1991; Wang et al., 2009), as far as we know, this is the first confirmed study to demonstrate significant increases in CRF mRNA expression in the POA, analogous to the

mammalian anterior hypothalamus, and in plasma ACTH levels following administration of 8-OH-DPAT in teleost fish. An earlier study by Höglund et al. (2002) did address this issue, but was unable to show a significant increase in plasma ACTH in response to 8-OH-DPAT injection in Arctic charr despite increases in plasma cortisol. Interestingly, these investigators did find that when Arctic charr were stressed, activation of the 5-HT_{1A} receptor with 8-OH-DPAT resulted in an attenuation of the stress response at the level of cortisol secretion, with a tendency to decrease plasma ACTH levels, instead of a stimulation of the HPI axis (Hoglund et al., 2002). This finding led the group to concur with a theory put forth by Welch et al. (1993) that 5-HT_{1A} receptors located in the central nervous system (CNS) are responsible for inhibiting the stress axis, while secondary peripheral effects must mediate its stimulation via a cardiovascular/sympathomedullary reflex response and an increase in catecholamines. In the present study, crowded fish did experience a significant decrease in plasma ACTH levels compared to uncrowded fish after administration of 8-OH-DPAT. However, this could also be due to cortisol mediated downregulation of the 5-HT_{1A} receptor, a theory supported by the decrease in whole brain 5-HT_{1A} receptor mRNA levels and maximum binding observed in the study conducted by Medeiros and McDonald (2012). It is possible that the contradictions between the two fish studies could also be due to differences in dose, administration and sampling protocol. Alternatively, there could be interspecific differences in the receptor binding kinetics of pre- and postsynaptic variants of the 5-HT_{1A} receptor that may result in a combination of stimulatory (Polter and Li, 2010; Sullivan et al., 2005) and inhibitory (Mauk et al., 1988; Sprouse and Aghajanian, 1987; Sprouse and Aghajanian, 1988) effects.

When comparing the partial amino acid sequence of the Gulf toadfish CRF precursor to the relevant portion of the human homologue, it is clear that the majority of the gene has been highly conserved, especially in regards to the region that is cleaved off and becomes the CRF peptide. This similarity is important when we considered using the mammalian drug, antalarmin, which antagonizes the CRF-1 receptor, preventing CRF-stimulated ACTH secretion (Tsigos and Chrousos, 2002; Willenberg et al., 2000). Until recently there was no way to pharmacologically manipulate *in vivo* CRF-mediated ACTH secretion; separating the effects of activating 5-HT_{1A} receptors which increase CRF concentrations in the POA from those located in the pituitary required a hypophysectomy, a highly invasive procedure. Antalarmin has been used successfully in another teleost study (Lastein et al., 2008). In the present study of toadfish, antalarmin prevented CRF-1 receptor-mediated ACTH secretion as Tween 80-injected, but not antalarmin-injected, fish showed a significant increase in plasma ACTH following administration of CRF via a caudal catheter. These results confirmed that both the CRF peptide and the CRF-1 receptor in Gulf toadfish are similar to the human homologues, adding to the mounting evidence for a highly conserved stress system between non-mammals and mammals (Chang and Hsu, 2004; Flik et al., 2006; Lastein et al., 2008; Medeiros et al., 2010).

In regards to corticosteroid secretion, groups working on mammals have debated the importance of the hypothalamus versus the pituitary for some time, with contradictory results (Calogero et al., 1989; Jørgensen et al., 2002; Simpson and Waterman, 1988). In the case of the Gulf toadfish, 5-HT_{1A} receptor mRNA expression studies certainly implied that the midbrain region, possessing 2.7-fold higher 5-HT_{1A} receptor transcript

than measured in the pituitary, might have greater impact on the release of cortisol (Medeiros et al., 2010). After confirming that 8-OH-DPAT caused a significant increase in POA CRF mRNA expression as well as plasma ACTH levels, antalarmin treatment allowed for us to pharmacologically separate the role of the 5-HT_{1A} receptor in increasing CRF concentrations in the POA from its role in directly stimulating an increase in ACTH secretion from the pituitary. In toadfish, both vehicle- and antalarmin-treated groups showed a significant increase in plasma [ACTH] following intravenous injection with 8-OH-DPAT. This is the logical outcome should populations of 5-HT_{1A} receptors in the telencephalon and preoptic regions as well as in the pituitary be separately contributing to 5-HT_{1A} receptor-mediated increases in total plasma ACTH. In fact, 5-HT_{1A} receptors in the pituitary appear to be playing an even bigger role in the regulation of ACTH secretion than the 5-HT_{1A} receptors causing increases in CRF in the POA since removing that component had no significant effect on circulating ACTH levels. While this finding is in agreement with other studies in teleosts (Young, 1993), it is contrary to a mammalian study by Jørgensen et al. (2002). This study used both 5-HT and fluoxetine, which inhibits the reuptake of serotonin from the neuronal synapse, to sustain high synaptic concentrations of 5-HT for as long as possible to exaggerate any response, should one occur. They determined that the increase in both CRF and ACTH mediated by 5-HT + fluoxetine treatment was inhibited when CRF antiserum was administered prior to treatment. Again, this discrepancy could be the result of different methodologies; 5-HT + fluoxetine were administered via an IP injection in Jørgensen et al. (2002). Alternatively, differences in results could be due to the fact that only 5-HT +

fluoxetine was used instead of 8-OH-DPAT, the latter having a higher affinity for the 5-HT_{1A} receptor than the former.

Mammalian studies have shown that chronic elevations in plasma cortisol levels activate a canonical negative feedback loop to inhibit the hypothalamic-pituitary-adrenal (HPA) axis; the mammalian homologue of the HPI axis. Inhibition occurs on all levels of the HPA axis and involves both long-loop and ultra-short-loop feedback regulatory mechanisms to decrease the release of CRF, ACTH, and corticosteroids (reviewed by Tsigos and Chrousos, 2002). The inhibitory effects of cortisol on the stress axis have also been observed in teleosts. Fryer and Peter (1977) first showed that cortisol implants suppress the synthesis and/or release of CRF in the hypothalamus of goldfish. In a subsequent study, Fryer et al. (1984) demonstrated that cortisol inhibited the release of ACTH from goldfish pituitary cells. On the level of the interrenal cells, Bradford et al. (1992) used an *in vitro* head kidney preparation to prove that the presence of cortisol in the incubation media was directly inhibiting the secretion of cortisol from the interrenal cells via an ultra-short-loop feedback mechanism.

Due to its involvement in the stimulation of the stress axis, the 5-HT_{1A} receptor was quickly discovered to also be a target of the feedback mechanisms. In mammals, corticosteroids have been found to downregulate transcription and binding kinetics of the 5-HT_{1A} receptor (Beer et al., 1990; Blier and de Montigny, 1987; Fanelli and McMonagle-Strucko, 1992; Gobbi et al., 1991; Kennett et al., 1987; Schechter et al., 1990). We have discovered a similar trend in the Gulf toadfish (Medeiros and McDonald, 2012) and believe that cortisol is mediating a decrease in the transcription of 5-HT_{1A} mRNA in the brain, which correlates with a decrease in maximum binding

(B_{max}). As it was possible to abolish the effects of chronic stress using the glucocorticoid receptor (GR) antagonist RU486, we believe that the downregulation in 5-HT_{1A} is mediated by GR (Medeiros and McDonald, 2012). These previous results combined with the direct involvement of the 5-HT_{1A} receptor in the secretion of both CRF and ACTH, prompted us to investigate how crowding stress affected 5-HT_{1A} receptor-mediated secretion of both hormones and if the hypothesized downregulation due to crowding stress could be alleviated with RU486 treatment. Following our previous findings, treatment with RU486 appeared to allow for an increased secretion of CRF and ACTH in response to 8-OH-DPAT compared to crowded + vehicle treated fish. This likely means that the chronic stress of crowding is inhibiting the 5-HT_{1A} receptor-mediated release of CRF and/or ACTH through a downregulation in 5-HT_{1A} receptor mRNA and/or protein levels as described by Medeiros et al. (2012) and that the downregulation is reversed when inhibiting GRs by RU486. This reasoning is especially clear when considering that toadfish held in uncrowded conditions respond to 8-OH-DPAT injection in the same magnitude as crowded + RU486-treated fish. Taken together, it appears that chronically elevated cortisol, mediated by the GR, activates a negative feedback mechanism that inhibits 5-HT_{1A}-mediated CRF and ACTH secretion. In turn, this would likely attenuate cortisol secretion from the interrenal cells of the Gulf toadfish (Medeiros et al., 2010).

In summary, this study is the first teleost study to demonstrate that the 5-HT_{1A} receptor agonist, 8-OH-DPAT, stimulates a significant increase in the release of CRF from the POA and ACTH from the pituitary, with 5-HT_{1A} receptors in the pituitary perhaps playing a more important role. We have also provided evidence that cortisol, acting via GR, attenuates the 5-HT_{1A} receptor-mediated secretion of both CRF and

ACTH. Future studies will need to investigate the potential role of 5-HT and 5-HT_{1A} receptors at the level of the interrenal cells and determine if a GR-mediated negative feedback loop exists there as well.

CHAPTER 5
CORTISOL ATTENUATES ADRENOCORTICOTROPIN HORMONE- AND SEROTONIN-STIMULATED CORTISOL SECRETION FROM THE INTERRENAL CELLS OF THE GULF TOADFISH (*OPSANUS BETA*)

Background & significance

The involvement of the hypothalamus and pituitary gland in the control of glucocorticoid secretion is well established for many vertebrates, with corticotropin-releasing factor (CRF) and adrenocorticotropic hormone (ACTH) being the most important secretagogues and cortisol or corticosterone the main end products of the brain-pituitary-adrenal axis (Chrousos and Gold, 1992). This general vertebrate pattern also applies to teleosts (Sumpter et al., 1994); however, in fish, corticosteroid production is located in the interrenal cells. These cells do not form a compact gland comparable to the adrenal cortex, but are instead located around the walls of the posterior cardinal veins and its branches as it runs through the head kidneys (Chester Jones, 1980). The homologous neuroendocrine system in fish is thus known as the hypothalamic-pituitary-interrenal (HPI) axis.

When either serotonin (5-hydroxytryptamine; 5-HT) or the 5-HT_{1A}-specific agonist 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT) bind to the Gulf toadfish (*Opsanus beta*) 5-HT_{1A} receptor, they result in the release of CRF and ACTH from the POA/anterior hypothalamus and pituitary, respectively, and a significant elevation in plasma cortisol (Medeiros et al., 2010; Medeiros and McDonald, 2012; Winberg et al., 1997). Thus, 5-HT plays a part in regulating the secretion of cortisol as it is both directly and indirectly responsible for cortisol secretion, depending on the location and type of receptor. The 5-HT_{1A} receptor is distributed throughout the Gulf toadfish, though it is

concentrated in the brain and pituitary, which collectively possess at least twice as much 5-HT_{1A} mRNA transcript as any other tissue sample tested (Medeiros et al., 2010). Within the brain, the highest levels of expression are found in the midbrain and diencephalon (Medeiros et al., 2010), which contains the preoptic area (POA), a forebrain region in fish and mammals that contains a high concentration of CRF-secreting neurons, and the pituitary. In toadfish, the mRNA distribution of the 5-HT_{1A} receptor within the midbrain/diencephalon and pituitary correlates well with the 5-HT_{1A} receptor-mediated release of CRF and ACTH (Medeiros et al., 2012). As there is little expression of the 5-HT_{1A} receptor in the kidney (Medeiros et al., 2010), it is not likely that this receptor is responsible for directly stimulating the release of cortisol in fish from the interrenal cells; however, to our knowledge, 5-HT-induced cortisol secretion from fish interrenal cells has never been examined.

Cortisol is involved in a variety of functions, including regulation of energy metabolism, hydromineral balance, and immune competence (Wendelaar Bonga, 1997). Additionally, cortisol also acts as a self-suppressant, exerting negative feedback on all levels of the HPI axis to attenuate the stress response (Bradford et al., 1992; Medeiros et al., 2012; Medeiros and McDonald, 2012; Wendelaar Bonga, 1997). Cortisol exerts its effects by crossing the cell plasma membrane and binding to intracellular proteins called glucocorticoid receptors (GR) (Mommsen, 1999), which are expressed in nearly every vertebrate cell type (Kudielka et al., 2006). Immunohistochemistry studies on trout (Tujague et al., 1998) revealed GR-immunoreactive cells in the POA and the pituitary (Fuxe et al., 1985), supporting the idea of a long-loop negative feedback mechanism in which CRF and ACTH secretion are downregulated by corticosteroids (Fryer and Peter,

1977; Rotllant et al., 2000a; Rotllant et al., 2000b). Long-loop negative feedback against the 5-HT_{1A} receptor is evident in Gulf toadfish wherein crowding stress decreased the amount of brain 5-HT_{1A} receptor protein, as measured by maximum binding (B_{max}), as well as reduced the amount of 5-HT_{1A} receptor-mediated CRF and ACTH secretion from the POA and pituitary, respectively (Medeiros et al., 2012; Medeiros and McDonald, 2012). GRs are most likely responsible for mediating this decrease, as treatment with the GR antagonist, RU486, restored 5-HT_{1A} mRNA expression levels, B_{max} and 5-HT_{1A} receptor-mediated CRF and ACTH secretion levels to control values (Medeiros et al., 2012; Medeiros and McDonald, 2012). Even though RU486-treatment abolished the effects of crowding stress on 5-HT_{1A} mRNA levels, B_{max} and function in the brain, *in vivo* 5-HT_{1A} receptor-induced cortisol secretion was still suppressed, suggesting the involvement of a non-GR-mediated, ultra-short loop, negative feedback mechanism on cortisol release directly at the interrenal cells. Supporting this theory, Bradford et al. (1992) demonstrated that cortisol induces self-suppression by ultra-short-loop negative feed-back in isolated head kidney of coho salmon. The same suppression of 5-HT_{1A} receptor-mediated cortisol release was observed in cortisol-implanted fish (Medeiros et al., 2012), perhaps suggesting that the increased circulating cortisol associated with RU486 treatment was involved.

Thus, both long-loop and ultra-short-loop feedback regulatory mechanisms of the HPI axis have been documented in fish. However, studies of the neuroendocrine control of the HPI axis in fish have focused on long-loop regulatory mechanisms; the existence of ultra-short-loop feedback has not yet been thoroughly documented nor has a mediator for negative feedback been identified. Additionally, while 5-HT triggers cortisol

secretion in fish by activating the HPI axis at the level of the CNS, it is not known if the interrenal cells themselves secrete cortisol in response to stimulation with 5-HT. Using an *in vitro* approach, this study had three main goals. First, we attempted to determine if cortisol mediates a decrease in the sensitivity of Gulf toadfish interrenal cells to ACTH and 5-HT. Second, should a decrease in ACTH sensitivity be observed, we sought to conclude if there was a role for GRs in the attenuation of ACTH-stimulated cortisol secretion. Third, as both 5-HT-mediated CRF and ACTH secretion appear to be affected by stress at the level of the CNS, we attempted to resolve which, if any, 5-HT receptor was responsible for 5-HT-induced increases in cortisol secretion from the interrenal cells and if 5-HT-mediated cortisol secretion was affected by increased plasma cortisol levels.

Materials and methods

Experimental animals

Gulf toadfish (*Opsanus beta*) were captured by commercial shrimpers using roller trawls in Biscayne Bay, Florida in the summer and fall of 2011. After capture, fish were immediately transferred to the laboratory where they were held for up to one month. Upon arrival in the lab, fish were placed in fresh water for 5 minutes and then treated with a dose of malachite green (final concentration 0.05 mg l-1) in formalin (15 mg l-1) (AquaVet) to prevent infection by the ciliate, *Cryptocaryon irritans* (Stoskopf, 1993). The fish were kept in 50-liter glass aquaria with flowing, aerated seawater at a temperature of 20-22ºC and were fed weekly with squid.

Experimental Treatments

Series 1: Endogenous plasma cortisol elevation by crowding

Uncrowded toadfish (0.035 ± 0.001 kg, N = 16) were placed individually into minnow traps and separated into 4 outdoor 6000 L mesocosm tanks (6 fish per tank) seeded with the seagrass, *Thalassia testudinum,* emulating the natural environment of Gulf toadfish (Serafy et al., 1997). To test 5-HT agonists, 20 more fish (0.037 ± 0.002 kg) were placed in individual minnow traps. In addition, two separate groups of toadfish were crowded (10 fish per 10 L water) in the laboratory and left for one week before collection, with the smallest 9 fish sampled each time (0.034 ± 0.002 kg, N = 18) (to be consistent in terms of social status). To decrease the chance of handling stress confounding results, blood samples were drawn immediately after removing the fish from the water *via* ventral caudal puncture using a 23G needle attached to a disposable syringe as described by Medeiros et al. (2010). Each fish was sampled within a 5 minute period; this time period is short enough so that plasma cortisol levels are indicative of basal levels and not the result of sampling (Medeiros et al. 2010). Collected samples were centrifuged at 16,000 *g* for 10 minutes and the plasma decanted. Plasma from each sample was flash frozen in liquid nitrogen (N_2), and then stored at -80°C until used for the evaluation of circulating levels of cortisol. Whole kidneys were collected from fish first anesthetized with a lethal dose of MS-222 (3 $g \cdot L^{-1}$; Finquel). Individual kidneys were immediately placed in 1 mL of ice cold Liebovitz's L-15 with L-glutamine (L-15; Cellgro by MediaTech, Inc.) and kept on ice until being used in the kidney preparation (see *Experimental Protocols* below).

Series 2: Exogenous cortisol loading with coconut oil implants

To determine the effect of cortisol alone on the functional response of the interrenal cells without the social stress of crowding, fish were injected intraperitoneally with either 1 mL coconut oil·100 g fish^{-1} alone (vehicle fish [0.040 ± 0.002 (N = 17) kg]) or with cortisol (11β,17α,21-Trihydroxy-4-pregnene-3,20-dione 21-hemisuccinate sodium salt; Sigma Aldrich) at a dose of 10 mg cortisol·mL coconut oil·100 g fish^{-1} (cortisol-implanted fish [0.033 ± 0.001 (N = 17) kg]) as described by Vijayan et al. (1994) and Morando et al. (2009). Intraperitoneal implantation of cortisol using coconut oil as a vehicle has been shown to produce a slow release of cortisol into the circulation (Vijayan and Leatherland, 1989). After injection with the warmed coconut oil, an ice pack was placed against the site of injection to facilitate the solidification of the implant. Following implantation, fish were placed in individual 2 L tubs and left undisturbed for 48h. Blood samples were drawn and stored until assays were performed as described above. Kidneys were extracted (see *Series 1*) and kept in 1 mL of ice cold L-15 until the kidney preparation was performed (see *Experimental Protocols* below).

Series 3: Exogenous cortisol loading with pharmacological RU486 treatment

To investigate how the effects of cortisol on interrenal cell function were being mediated, fish were injected intraperitoneally with either cortisol (11β,17α,21-Trihydroxy-4-pregnene-3,20-dione 21-hemisuccinate sodium salt; Sigma Aldrich) at a dose of 10 mg cortisol·mL coconut oil·100 g fish^{-1} (cortisol-implanted fish [0.037 ± 0.002 (N = 5) kg]) or 10 mg cortisol·100 g fish^{-1} mixed with 100 mg RU486·100 g fish^{-1} in 1 mL coconut oil·100 g fish^{-1} (cortisol + RU486-implanted fish [0.037 ± 0.001 (N = 8) kg];

mifepristone; Sigma Aldrich). RU486 is commonly administered to Gulf toadfish at doses 10-fold greater than circulating cortisol concentrations to ensure that glucocorticoid receptors are adequately antagonized (McDonald et al., 2009; McDonald et al., 2004). Blood samples were drawn and stored as described above until assays were performed. Kidneys were extracted (see *Series 1*) and kept in 1 mL of ice cold L-15 until the kidney preparation was performed (see *Experimental Protocols* below).

Experimental protocols

Kidney preparation. After being excised, the entire kidney (containing the interrenal tissue) was finely cut into ~ 1 mm^3 pieces. The entire kidney was then transferred to one of the wells in a 24-well sterile culture plate (Corning Inc.) with 1 mL of fresh L-15, covered with tinfoil, and placed on an orbital plate rotator (Lab-Line) set at approximately 125 RPM. Tissue pieces were then pre-incubated at room temperature (approximately 25 °C) for 2 h in 1 mL L-15, with bath changes at 1 h and 1.5 h. After 2 h, a 35 μL sample of the pre-incubation media was taken to verify that the tissue was no longer spontaneously secreting cortisol. After the sample was taken, the tissue was washed with 1 mL L-15 for five minutes, after which time it was placed in the experimental incubation media. For *Series 1* the stimulants included in the experimental incubation media were: 5-HT (Sigma Aldrich) and adrenocorticotropin hormone (ACTH; Tocris Biosciences). Additionally, (±)-8-Hydroxy-*N*,*N*-dipropyl-2-aminotetralin (8-OH-DPAT; 5-HT_{1A} agonist; Sigma Aldrich), α-methylserotonin (a 5-HT_2 agonist; Sigma Aldrich), and RS67506 (a 5-HT_4 agonist; Tocris Biosciences) were also used as stimulants. Only 5-HT and ACTH were used as stimulants for *Series 2*. And, for *Series*

3, all kidneys were only stimulated with ACTH. Preliminary experiments determined the lowest effective concentration (LEC) of 5-HT and ACTH (from a range of 1.0×10^{-7} M to 1.0×10^{-5} M) that resulted in a significant increase in [cortisol] after 2 h to be 1.0×10^{-5} M and 3.3×10^{-7} M, respectively. Consequently, all experiments used these concentrations (with the 5-HT agonists used in *Series 1* being used at the same concentration as 5-HT itself). To avoid freezing and thawing the L-15 media, stock concentrations of pharmacological agents were made up in water and diluted with L-15; depending on the stock concentration, serial dilutions put the final water concentration at ≤ 1%. A 35 μL sample was taken from every well at 0.5, 1, 2, 3 and 4 h and then frozen in liquid nitrogen before being stored at -80 °C until analysis of cortisol and ACTH (MP Biomedicals).

Analytical Techniques. Plasma cortisol and ACTH were quantified using the respective MP Biomedical ^{125}I RIA kit, with the cortisol standards diluted by half so that protein concentrations were within the range measured in toadfish. ACTH standards were used as provided.

Statistics. Unless otherwise noted, analyses were conducted using SigmaStat and/or SigmaPlot (SPSS, Inc.). Data are given as means ± 1 s.e.m., and N = the number of fish in each assay. To determine differences amongst treatments, an ANOVA was performed followed by a Bonferroni post-hoc test when a significant difference was found ($p<0.05$).

Results

Initial experiments in uncrowded fish evaluated all time points to determine where a significant increase in cortisol secretion was observed. Analyses revealed that for both ACTH and 5-HT, a consistent and significant increase in cortisol excretion was observed after 2 h. Thus, only data at the 2 h time point is presented and analyzed.

The effect of endogenous cortisol elevation on Gulf toadfish interrenal cell function was evaluated by comparing fish that were stressed by crowding to fish that were held in uncrowded conditions. Plasma cortisol levels were, on average, 7.5-times higher in crowded toadfish compared to the uncrowded controls ($p < 0.001$; Table 5.1). After 2 h, interrenal cells from uncrowded Gulf toadfish secreted 53.5 ± 13.9 and 7.4 ± 3.6 ng of cortisol·50 mg wet kidney tissue^{-1} in response to stimulation with ACTH and 5-HT, respectively, which was 76.6-times and 10.6-times more than the amount of cortisol secreted by unstimulated interrenal cells ($P< 0.001$ and $P < 0.05$, respectively; Fig. 5.1). The unstimulated interrenal cells taken from crowded toadfish secreted 1.98 ± 0.52 ng of

Table 5.1. Plasma cortisol levels of treatment groups.

Treatment	Plasma cortisol (ng·mL^{-1})	N
Uncrowded	23.66 ± 5.54	41
Crowded	176.86 ± 26.22*	18
Vehicle-Implanted	278.41 ± 26.60	15
Cortisol-Implanted	1186.93 ± 55.54$^{\#}$	20
Cortisol + RU486-Implanted	1411.05 ± 190.02$^{\#}$	8

Values are mean ± S.E.M.; * $P < 0.001$ compared to uncrowded, $^{\#}$ $P < 0.001$ compared to vehicle-implanted.

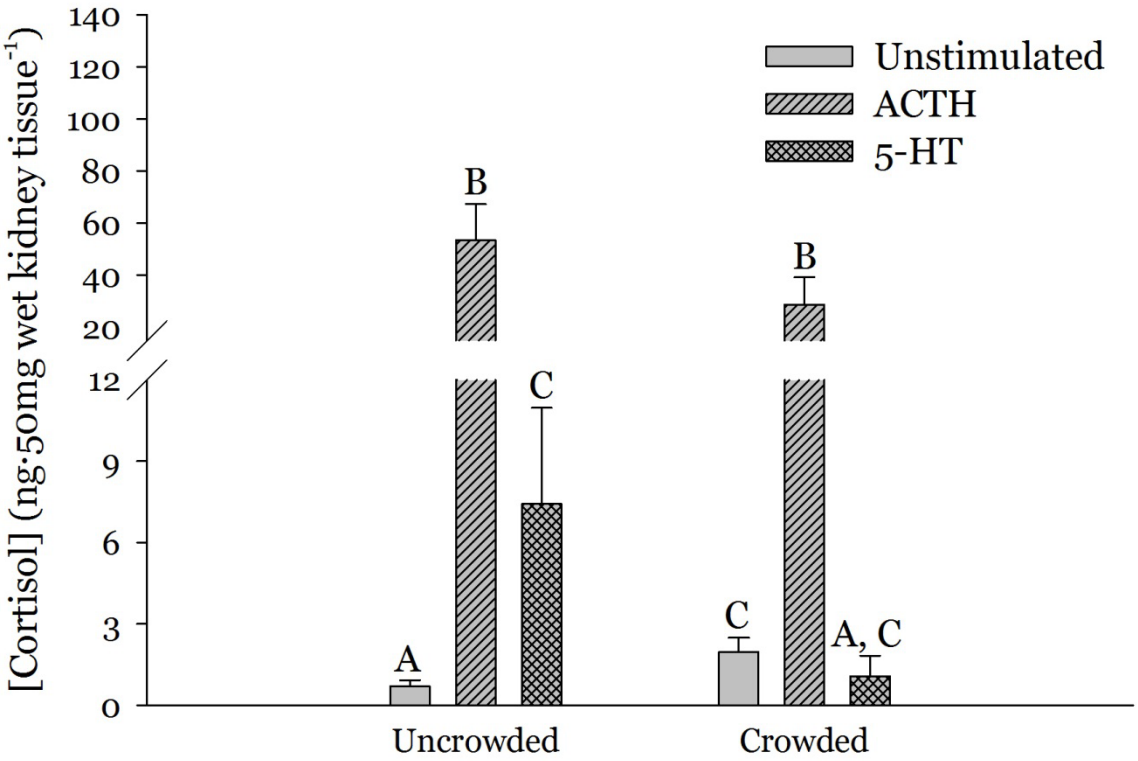

Figure 5.1. *In vitro* cortisol release from *in vitro* Gulf toadfish kidneys following 2 h of stimulation with L-15 media (unstimulated), ACTH in L-15, or 5-HT in L-15 taken from either uncrowded or crowded fish. Different letters represent a significant difference between treatment groups ($P < 0.05$) and bars represent the mean ± s.e.m. ($N = 6$, except for the unstimulated and 5-HT uncrowded groups where $N = 10$).

cortisol·50 mg wet kidney tissue^{-1}, which was 185% more spontaneous cortisol secretion than that observed in the uncrowded treatment ($P < 0.05$; Fig. 5.1). Interrenal cells from crowded fish responded to stimulation with ACTH by increasing cortisol secretion 14.6-fold ($P < 0.05$; Fig. 5.1), which was not significantly different than the ACTH-stimulated cortisol secretion from interrenal cells taken from uncrowded fish; however, crowding appeared to have attenuated 5-HT-stimulated cortisol secretion ($P > 0.05$; Fig. 5.1).

Intraperitoneal (IP) cortisol implants were used to see if pharmacological plasma cortisol levels could attenuate the secretion of cortisol from the interrenal cells. Cortisol implants raised plasma cortisol levels to 1186.9 ± 55.5 ng·mL^{-1}, which was 4.5-times higher than the 278.4 ± 26.6 ng·mL^{-1} observed in the vehicle-implanted control group (P

< 0.001; Table 5.1). The unstimulated, vehicle-implanted group released a total of 8.3 ± 1.9 ng of cortisol·50 mg wet kidney tissue^{-1} after a period of 2h. When stimulated by ACTH, the total amount of cortisol secreted by the vehicle-implanted group increased by 12.3-fold to 102.0 ± 23.1 ng of cortisol·50 mg wet kidney tissue^{-1}, compared to the unstimulated kidneys (P < 0.01; Fig. 5.2). In the vehicle-implanted controls, 5-HT was

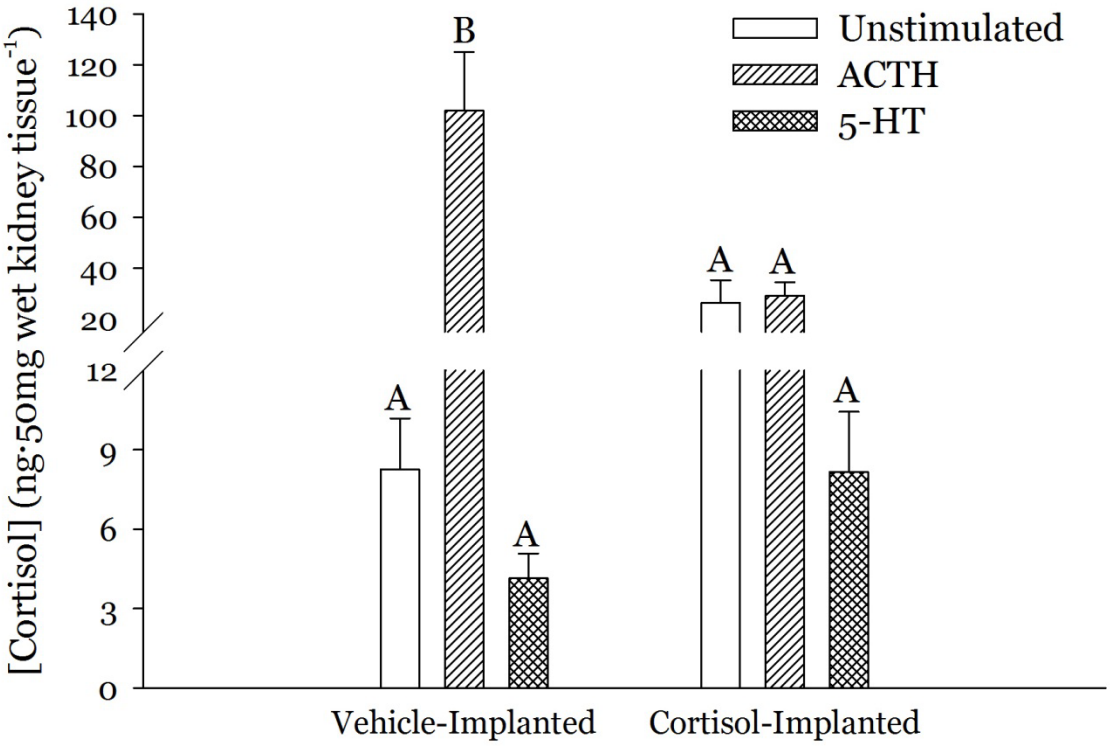

Figure 5.2. Amount of cortisol released after 2 h from *in vitro* Gulf toadfish kidneys in response to stimulation with L-15 media (unstimulated), ACTH in L-15, or 5-HT in L-15 removed from either vehicle- or cortisol-implanted fish. Different letters represent a significant difference between treatment groups (P < 0.05) and bars represent the mean ± s.e.m. (N = 5, except for the cortisol-implanted ACTH-stimulated group where N = 10).

not able to elicit a response that was significantly different than unstimulated kidneys (P > 0.05; Fig. 5.2). Interestingly, cortisol implants effectively attenuated ACTH-stimulated cortisol secretion, thus cortisol secretion by the unstimulated, ACTH-stimulated, and 5-HT-stimulated kidneys was not significantly different (P > 0.05; Fig. 5.2).

To elucidate how the increased circulating level of cortisol was mediating a change in cortisol secretion from the interrenal cells, the glucocorticoid receptor (GR) antagonist RU486 was utilized along with IP cortisol implants. Plasma cortisol was not significantly different between the cortisol- and cortisol + RU486-treated groups ($P > 0.05$; Table 5.1) and was similar to the circulating level measured in the cortisol-implanted fish from *Series 2*. Intriguingly, RU486 did not restore the ability of the interrenal cells to secrete cortisol in response to ACTH and, in response to stimulation with ACTH, kidneys from both cortisol and cortisol + RU486 treated fish secreted approximately 72.6% less cortisol than the vehicle-implanted fish ($P < 0.05$; Fig. 5.3).

To further investigate the role of 5-HT-stimulated cortisol release from the

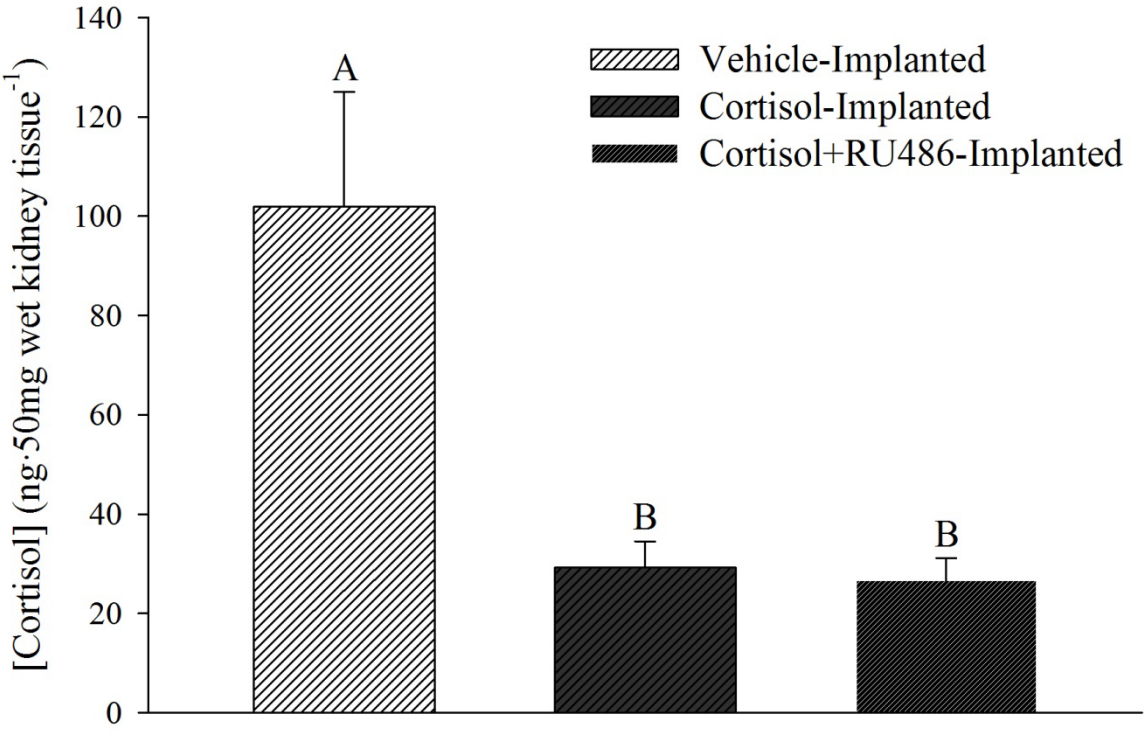

Figure 5.3. *In vitro* cortisol secretion measured from *in vitro* Gulf toadfish kidneys following 2 h of stimulation withACTH in L-15 isolated from fish subjected to 48 h of vehicle-, cortisol- or cortisol + RU486-implantation. Different letters represent a significant difference between treatment groups ($P < 0.05$) and bars represent the mean ± s.e.m. (N = 5 for the vehicle-implanted group, N = 10 for the cortisol-implanted group, and N = 8 for the cortisol + RU486-implanted group).

interrenal cells of the Gulf toadfish, 3 different 5-HT agonists were employed: α-methylserotonin (a general 5-HT_2 receptor agonist), 8-OH-DPAT (a highly specific 5-HT_{1A} receptor agonist), and RS67506 (a 5-HT_4 receptor agonist). After 2 h, both α-methylserotonin and RS67506 were able to elicit an 8.6- and 16.3-fold increase ($P < 0.05$; Fig. 5.4) in cortisol secretion compared to the unstimulated control. In contrast, the 5-HT_{1A} agonist, 8-OH-DPAT, was unable to elicit a significant increase in cortisol secretion compared to the unstimulated control and was significantly lower than cortisol secretion by interrenal cells when stimulated with α-methylserotonin or RS67506 ($P < 0.05$; Fig. 5.4).

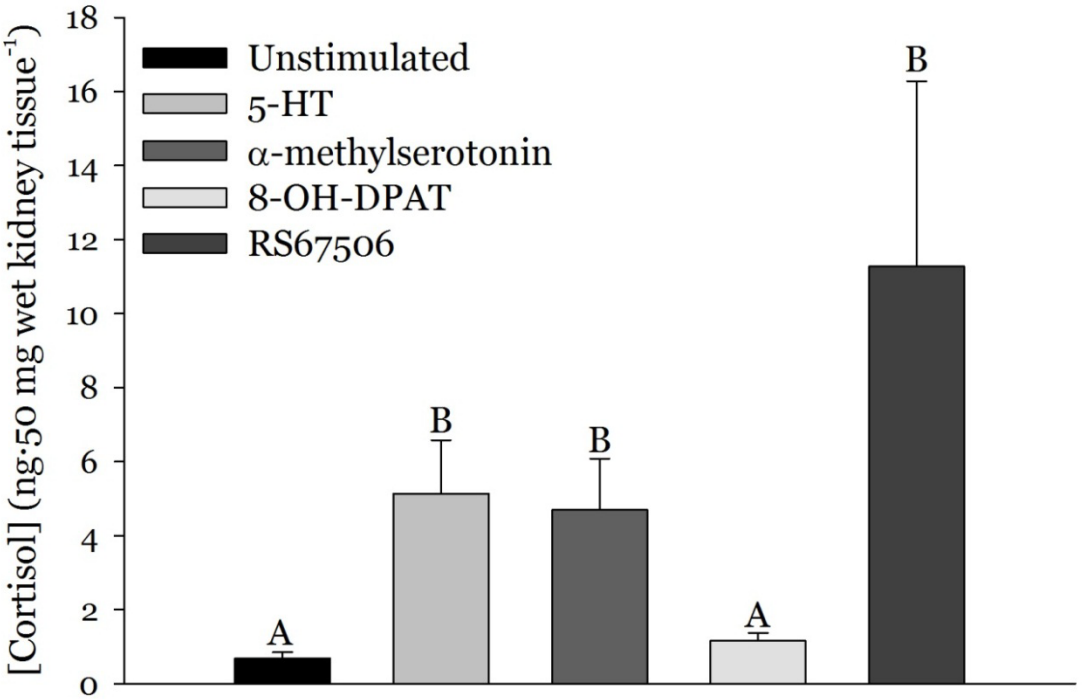

Figure 5.4. *In vitro* cortisol secretion from *in vitro* Gulf toadfish kidneys in response to 2 h of stimulation with L-15 media (unstimulated), 5-HT in L-15, α-methylserotonin in L-15, 8-OH-DPAT in L-15, or RS67506 in L-15 taken from uncrowded fish. Different letters represent a significant difference between treatment groups ($P < 0.05$) and bars represent the mean ± s.e.m. ($N = 5$, except for the uncrowded unstimulated group where $N = 10$).

Discussion

Adrenocorticotropic hormone (ACTH) is the main secretagogue responsible for cortisol secretion in fish (Donaldson, 1981), so it was no surprise that ACTH elicited a significant cortisol response from uncrowded Gulf toadfish kidney tissue. Similar to past studies, a significant increase in spontaneous cortisol secretion from head kidneys of crowded fish was measured in the Gulf toadfish. The increase in spontaneous cortisol secretion from unstimulated interrenal cells may be explained by alterations in cortisol clearance rates (Vijayan and Leatherland, 1990), hyperplasia of cortisol-producing interrenal cells, and/or the involvement of additional corticotropic factors. Crowding stress did not cause a significant decrease in ACTH-stimulated cortisol secretion when compared to the amount released by uncrowded controls, which is in contrast to the decrease in ACTH sensitivity following prolonged exposure to crowded conditions that has been observed in the gilthead sea bream (Sparus aurata; Rotllant et al., 2000b) and the brook charr (*Salvelinus fontinalis*; Vijayan and Leatherland, 1990). This may be due to an increased tolerance to cortisol in the Gulf toadfish compared to the gilthead sea bream and the brook charr. While the average uncrowded plasma cortisol levels are similar in all species (between 3 and 15 $ng \cdot mL^{-1}$), the Gulf toadfish seems to have a markedly higher cortisol response to acute stress with up to twice the amount of cortisol being secreted following stimulation of the HPI axis (Biron and Benfey, 1994; Medeiros et al., 2010; Rotllant et al., 2001).

Elevation of circulating cortisol levels through cortisol implants raised circulating cortisol levels to > 1000 $ng \cdot mL^{-1}$ *in vivo* and significantly attenuated ACTH-stimulated cortisol secretion from *in vitro* isolated kidney tissue while also revealing a tendency for

elevated spontaneous cortisol secretion. Interestingly enough, the attenuation of ACTH-stimulated cortisol secretion was observed following incubation in a cortisol-free experimental media, suggesting that the attenuation measured is the result of a previously established change, perhaps the result of the chronically elevated cortisol levels, which is presenting as a decrease in sensitivity to ACTH. This decreased sensitivity to ACTH could be the result of a downregulation or desensitization of the melanocortin subtype 2 (MC_2) receptor, which binds to ACTH and initiates the production and secretion of cortisol.

The mechanism by which the decrease in interrenal ACTH sensitivity is mediated was investigated using the glucocorticoid receptor (GR) antagonist RU486 that has been shown to effectively block the negative feedback effects of elevated cortisol (Bernier et al., 1999; McDonald et al., 2009; McDonald et al., 2004; Rodela et al., 2011). Previous studies on toadfish demonstrated that cortisol elevation resulted in a decrease in 5-HT_{1A} receptor mRNA expression and binding as well as 5-HT_{1A} receptor-mediated CRF, ACTH and, ultimately, cortisol secretion (Medeiros et al., 2012; Medeiros and McDonald, 2012). Interestingly, treatment with RU486 alleviated the effects of elevated cortisol levels on transcript and protein levels of the 5-HT_{1A} receptor as well as 5-HT_{1A} receptor-mediated CRF and ACTH secretion, but it did not restore cortisol secretion (Medeiros and McDonald, 2012), suggesting that the inhibition of cortisol secretion is not GR-mediated at the level of the interrenal tissue. Thus, it was not surprising that kidney tissue excised from cortisol + RU486-treated fish experienced a reduction in ACTH sensitivity that was not GR-mediated. A decrease in ACTH sensitivity following exposure to elevated levels of cortisol, either *in vivo* or *in vitro*, has been observed in

other teleost species (Bradford et al., 1992; Rotllant et al., 2000b; Rotllant et al., 1997; Vijayan and Leatherland, 1990); however, no other study has investigated the role of the GRs in this response. Instead, these other studies suggested that the decrease may be explained by alterations in cortisol clearance rates (Vijayan et al., 1990), downregulation of the MC_2 receptor, a decrease in the activity of dibutyryl cyclic AMP (Patino et al., 1986), and/or the involvement of additional corticotropic factors, such as melanocyte-stimulating hormone (Rotllant et al., 2000b; Vijayan and Leatherland, 1990). Considering how conserved the stress response is among vertebrate taxa, it is also likely that neuropeptide Y (NPY), which is known to be present in fish (Volkoff et al., 2005), may act as a paracrine factor responsible for blunting the interrenal responses to ACTH stimulation in teleosts, as suggested by Wolfensberger et al. (1995). Precedence for this has been found in mammalian studies; Kosti et al. (2006) found that Maudsley reactive rats, which present with elevated anxiety-like behavior, were less responsive to ACTH compared to the less anxious Wistar control strain. After investigating several possible intra-adrenal regulators, the only significant molecular difference in the adrenal glands from the two strains was the level of expression of neuropeptide Y (NPY), which is known to be a stress-responsive peptide in the adrenal gland (Nussdorfer and Gottardo, 1998). Alternatively, the change in synthetic capacity could also be the result of steroid end products behaving as pseudosubstrates, bringing about a lipid peroxidation reaction that destroyed 11β-hydroxylase activity, as suggested by Hornsby (1980). Further investigation is needed to confirm these theories for teleosts.

Serotonin stimulated a significant elevation in cortisol release, though, as the magnitude of cortisol release in response to 5-HT was only 12.6% of the amount released

in response to ACTH, its role is minor compared to ACTH. The insensitivity of the Gulf toadfish kidney to 8-OH-DPAT, a 5-HT_{1A} receptor agonist, confirmed that a 5-HT receptor other than the 5-HT_{1A} receptor mediates cortisol secretion at the level of the interrenal cells. This supports the theory suggested by Medeiros et al. (2010) who concluded as much based upon the low level of 5-HT_{1A} mRNA transcript observed in the kidney of uncrowded Gulf toadfish. While 8-OH-DPAT failed to stimulate the release of cortisol from isolated kidney tissue, both α-methylserotonin, a general 5-HT_2 receptor agonist, and RS67506, a 5-HT_4 receptor agonist, stimulated a significant amount of cortisol to be secreted compared to unstimulated tissue. The 5-HT_4 receptor has previously been found to stimulate the release of cortisol at the level of the adrenal cortex in both mammals (Contesse et al., 1994; Lefebvre et al., 1992) and frogs (Delarue et al., 1988), so the fact that it appears the 5-HT_4 receptor also plays a role in teleosts is no surprise; however, the fact that α-methylserotonin also stimulated cortisol secretion is a little surprising. As a general 5-HT_2 agonist, α-methylserotonin has been shown to bind to all three known 5-HT_2 receptor subtypes in mammals (Fuller, 1996). In mammals, both 5-HT_{2A} and 5-HT_{2C} receptor subtypes (but not 5-HT_{2B}) are associated with corticosterone secretion in rats (Leysen, 1991; Mikkelsen et al., 2004); however, Mager et al. (submitted) showed that 5-HT_{2A} expression in the kidney of the Gulf toadfish is not significantly different from zero. This implies that, of the 5-HT receptor subtypes investigated, both the 5-HT_{2C} and 5-HT_4 receptors are most likely responsible for serotonin-mediated cortisol secretion in Gulf toadfish.

Crowding, vehicle-implanted and cortisol implanted fish displayed an inhibition of 5-HT-induced cortisol secretion when compared to the unstimulated crowded controls.

This was due to both a tendency for 5-HT-mediated cortisol release to be lower than in uncrowded fish and also the increase in spontaneous cortisol secretion from the kidney tissue of crowded fish. Based on the aforementioned results obtained by testing with various 5-HT receptor agonists, this is most likely the result of a decrease in mRNA transcript and/or binding kinetics of the 5-HT_{2C} or 5-HT_4 receptor, though there is no supporting evidence in the literature that connects stress to such changes in 5-HT_{2C} or 5-HT_4 receptors. In fish, like in other vertebrates, behavioral and physiological stress responses are linked by common control mechanisms in the brain, and two of the monoamine neurotransmitters associated with these receptors (5-HT and dopamine) play vital roles in this coordination (Höglund et al., 2002; Larson et al., 2003b; Perreault et al., 2003; Winberg et al., 1997; Winberg and Nilsson, 1993). While it is obvious how 5-HT, as the natural agonist, is involved in modulating the 5-HT_{2C} and 5-HT_4 receptors, it is not immediately clear how dopamine is associated. Dopamine, a monoamine in the catecholamine family, is responsible for many functions, including many social behavior disorders, and mammalian studies have associated its release with both the 5-HT_{2C} (Egerton et al., 2008; Leggio et al., 2009; Scarlota et al., 2011) and 5-HT_4 receptors (De Deurwaerdere et al., 1997; Thorre et al., 1998). Furthermore, as both 5-HT_{2C} and 5-HT_4 receptors are associated with anxiety in mammals (Christianson et al., 2010; Kennett et al., 1997), it is very likely that a stressful social situation, such as experienced by fish during crowding, would affect the function of these receptors in an attempt to attenuate the stress response.

In summary, we were able to demonstrate that crowding and exogenous cortisol were both able to affect HPI axis regulation to differing degrees, possibly correlating with

plasma cortisol level, and that both treatments resulted in a significant increase in spontaneous cortisol secretion from the interrenal cells, which correlates with increased circulating levels of cortisol. We also demonstrated that 5-HT has the ability to stimulate the release of cortisol from the interrenal tissue of a teleost fish and that the 5-HT_{2C} and 5-HT_4 receptors likely play a role in regulating this function and that 5-HT-induced cortisol secretion is more sensitive to circulating levels of cortisol than is ACTH-stimulated cortisol release. This is an interesting result that could be related to social status and merits further investigation to elucidate a role for serotonin in basal cortisol levels.

CHAPTER 6:
CONCLUSIONS & IMPLICATIONS

The series of investigations outlined in this dissertation have provided key information about the functional aspects of the 5-HT_{1A} receptor in a single species, and of the role it plays in both activating and attenuating the stress response in the Gulf toadfish (*Opsanus beta*). Time and again it was demonstrated that the pharmacology and functionality of the Gulf toadfish 5-HT_{1A} receptor were very similar to characteristics determined in mammalian studies; however, some key differences were also observed.

In mammals, particularly high expression of 5-HT_{1A} is found within the hippocampus, pituitary, and raphe nuclei (Kumar and Mann, 2007; Passchier et al., 2000). Distribution of 5-HT_{1A} transcript throughout the brain varied greatly in toadfish, though the expression pattern is congruent with mammalian studies. The brain region defined as the toadfish midbrain and diencephalon section (see Fig. 2.5), contained a significantly higher level of 5-HT_{1A} mRNA transcripts than all other regions. Given the relatively high conservation of the serotonergic system in vertebrates, the high expression of 5-HT_{1A} receptors found in this region indicates that the region (as defined by the authors) includes the dorsal raphe nuclei and hippocampal primordium (analogous to the hippocampus in mammals), as the 5-HT_{1A} receptor is present only in low levels in the hypothalamus of humans and other mammals (Kumar and Mann, 2007; Passchier et al., 2000). This implies that the 5-HT_{1A} receptor is involved in activating the stress response early on, and, as the pallial region is analogous to the hippocampus, could even be involved in processing how stimuli are perceived by the organism.

Early work postulated that activation of the 5-HT_{1A} receptor inhibited firing of the postsynaptic cell as 5-HT exerted a predominantly inhibitory effect on neuron firing rate in many areas of the brain (Araneda and Andrade, 1991; Clark et al., 1987; Davies et al., 1987; Kow et al., 1992; Newberry and Priestley, 1988). More recently, it has been discovered that there are two types of 5-HT_{1A} receptors: somatodendritic autoreceptors and postsynaptic receptors. Stimulation of 5-HT_{1A} autoreceptors in the raphe nuclei has an anxiolytic, or anti-anxiety effect while postsynaptic 5-HT_{1A} receptors located in the projection areas of the raphe nuclei, such as the hippocampus and hypothalamus, have an anxiogenic, or anxiety-promoting, effect when activated (File et al., 1996). Additionally, tracing studies have revealed that the 5-HT neurons involved in the secretion of CRF originate in the dorsal and medial raphe nuclei and project to the parvocellular division of the paraventricular nucleus, where they form synaptic connections with CRF-secreting neurons (Larsen et al., 1996). 5-HT_{1A} mRNA expression in the Gulf toadfish brain revealed relatively high levels in both the hindbrain and diencephalon/midbrain regions. The hindbrain contains the medulla oblongata, where the caudal raphe nuclei are located in both mammals and fish (Nieuwenhuys et al., 1998). Additionally, based on the expression pattern in mammals and the analogous structures in fish, it is likely that the midbrain/diencephalon region (as defined in Fig. 2.5) encompasses the hippocampus and dorsal raphe nuclei. Taken together, this implies that both somatodendritic autoreceptors and postsynaptic receptors are present in the Gulf toadfish; however, based on the tracing study by Larsen et al. (1996), it is likely that only those located in the midbrain/diencephalon region are involved in the stress axis of toadfish.

In mammals, it is well established that corticosteroids decrease 5-HT_{1A} receptor mRNA levels and protein binding (Beer et al., 1990; Blier and de Montigny, 1987; Fanelli and McMonagle-Strucko, 1992; Gobbi et al., 1991; Kennett et al., 1987; Schechter et al., 1990), but there are conflicting reports of where this downregulation occurs and also how it is mediated (*i.e.*, the glucocorticoid receptor or the mineralocorticoid receptor). Some studies indicate that it happens in the hippocampus (Meijer and de Kloet, 1994; Mendelson and McEwen, 1992), some find that it is limited to the somatodendritic autoreceptors located in the caudal raphe nuclei (Beer et al., 1990; Blier and de Montigny, 1987; Fanelli and McMonagle-Strucko, 1992; Gobbi et al., 1991; Kennett et al., 1987; Schechter et al., 1990; Seckl and Fink, 1991), while still others determine it to occur in both regions (de Kloet et al., 1986). Interestingly, none report a change in the pituitary, making the results of Chapter 4 to be the first we know of to demonstrate a stress-related decrease in the function of 5-HT_{1A} receptors located in the pituitary. However, while ACTH secretion was restored following treatment with RU486, the methodology does not allow us to state that the stress-related decrease in ACTH secretion from the pituitary was mediated by GRs, just that GRs mediate a significant, cortisol-induced decrease in ACTH secretion. Due to the emerging evidence concerning the presence and opposing functionality of the two 5-HT_{1A} receptor populations, this study could have benefited from a more in-depth investigation of 5-HT_{1A} mRNA levels using different brain segments from toadfish with elevated cortisol levels (as opposed to whole brains). Also, utilizing recently developed agonists that differentiate between the two populations for the whole brain binding assays would have helped determine a clear role for each in terms of HPI axis regulation. Additionally,

using these techniques may have helped elucidate discrepancies between mRNA levels and protein binding should the different receptor types have opposite responses to increased cortisol levels. Based on the results from the studies conducted for this dissertation, it is likely that elevated cortisol would have caused decreases in the activity of both types of 5-HT_{1A} receptors.

The results of Chapter 4 will also add fuel to the fire of a debate on the relative importance of the hypothalamus and the pituitary in regulating the release of corticosteroids. Treatment with the CRF-1 receptor antagonist, antalarmin, did not significantly decrease 8-OH-DPAT-stimulated ACTH secretion in the Gulf toadfish suggesting that the pituitary may actually play a larger role in total 5-HT_{1A}-stimulated ACTH secretion, and thus cortisol release. This finding was contrary to that of a mammalian study by Jørgensen et al. (2002), which did find a significant decrease in 5-HT-stimulated ACTH secretion following pretreatment with CRF antiserum, suggesting that, in rats, the 5-HT + fluoxetine-mediated increase in plasma ACTH is at the level of the hypothalamus and not the pituitary. The group used both 5-HT and fluoxetine, which inhibits the reuptake of 5-HT from the neuronal synapse, as a way to sustain high synaptic concentrations of 5-HT for as long as possible to exaggerate any response, should one occur. The discrepancy between the two studies could be the result of different methodologies; 5-HT + fluoxetine were used instead of 8-OH-DPAT in the present study, the latter having a higher affinity for the 5-HT_{1A} receptor than the former or it could also be due to the fact that 5-HT + fluoxetine were administered via an IP injection in Jørgensen et al. (2002) versus the intravenous method employed in our investigations. Additionally, as Fryer and Peter (1997) concluded that the hypothalamus

appears to have more control over corticosteroid release in the goldfish, it could be that the relative importance of the hypothalamus or the pituitary is species-dependent. Due to the fact that the pituitary plays a larger role in terms of 5-HT_{1A} receptor-mediated ACTH stimulation than the hypothalamus, it is possible that toadfish can provide a unique perspective on the mammalian stress axis and would perhaps be a useful animal to explore diseases involving dysfunctional endocrine regulation.

The investigations outlined in this dissertation also uncovered some interesting discoveries that are specific to the Gulf toadfish, though have exciting implications in general, in terms of nitrogen excretion, and also depression. A current theory in nitrogen, and specifically urea, excretion in toadfish is that the periodic activation or insertion of a specialized urea transporter that results in pulses of urea into the water is triggered by 5-HT (Wood et al., 2003; McDonald and Walsh, 2004) and coincides with, what appears to be, a permissive drop in plasma cortisol levels (McDonald and Walsh, 2004). Cortisol, in the regulation of this mechanism, is hypothesized to exert an inhibitory effect on the 5-HT receptor involved in mediating the 5-HT induced stimulation of urea excretion (reviewed by McDonald et al., 2012). When cortisol levels naturally drop in the fish, that inhibition is lifted, and a urea pulse occurs. At the same time, whether 5-HT is also involved in regulating these natural fluctuations in circulating cortisol has always been questioned. While the 5-HT receptors involved in regulating both the urea pulsing event and plasma cortisol levels are still being investigated, it is clear that injection with α-methylserotonin, a 5-HT_2 receptor agonist, stimulates a urea pulse, an effect that is inhibited by ketanserin, a 5-HT receptor antagonist most specific for the 5-HT_{2A} receptor (McDonald and Walsh, 2004). While the pieces of the puzzle have still not been put

together, it is obvious that both 5-HT and cortisol are involved, and knowing that multiple 5-HT receptors are involved in cortisol secretion may implicate them as playing a role in the regulation of pulsatile urea secretion in the Gulf toadfish. Additionally, it has been demonstrated that pulsatile urea excretion may be used as some sort of social communication tool and/or predator avoidance strategy (Barimo and Walsh, 2006; Sloman et al., 2005). The results from the *in vitro* kidney experiments in this dissertation imply that 5-HT-stimulated cortisol secretion is more easily attenuated by cortisol than ACTH-stimulated cortisol secretion, and, as 5-HT is involved in social and behavioral responses (McDonald et al., 2011), there could be a connection between the 5-HT receptor controlling cortisol secretion in the interrenal cells and the 5-HT receptor involved in urea pulsing.

Another interesting result gained from these studies was that the 5-HT_{1A} receptor mRNA expression was relatively high (compared to other tissues) in the swim bladder muscle, swim bladder lining, and gonads of the Gulf toadfish. As toadfish use their swimbladder as a means of communication during the reproductive cycle, the observed 5-HT_{1A} mRNA expression pattern makes a direct connection between the receptor, reproduction and a reproductive behavior. Considering that the 5-HT_{1A} receptor is of particular importance to the etiology and treatment of depression due to its ability to regulate both pre- and postsynaptic neurotransmission in the 5-HT system (Blier and de Montigny, 1999; Lesch and Heils, 2000; Veenstra-VanderWeele et al., 2000) and that many antidepressants work by increasing the amount of 5-HT available for binding by 5-HT receptors leading to desensitization of the 5-HT_{1A} receptor, there could be a link between antidepressant consumption, reproductive axis function and the 5-HT_{1A} receptor.

Lending credence to this theory is the fact that as many as 60% of people taking antidepressants are reportedly suffering from some sort of treatment-induced sexual dysfunction (Gregorian et al., 2002). In order to help those affected by mental illness, there is a need to elucidate the connections between 5-HT_{1A} function and the endocrine axes responsible for stress and reproduction in an attempt to develop novel treatments that could combat the sexual side effects of antidepressants and return patients to a truly normal life-style.

The studies herein have incorporated molecular, cellular and physiological techniques to help elucidate the interactions between the serotonergic system and HPI axis to further our understanding of the vertebrate stress response. These findings, combined with the fact that cortisol is the predominant corticosteroid in both humans and fish, provide support for considering the Gulf toadfish as a model for the human 5-HT_{1A} receptor.

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