Existence and Distribution of Serotonin and its 5-HT_2-like Binding Site in the Brain:Pituitary Axis of Juvenile And Sexually Recrudescing Female Rainbow Trout (*Oncorhynchus mykiss*).

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DEDICATION

This thesis is dedicated to my family, to thank them for their infinite support and encouragement.

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ABSTRACT

In the first phase of this study, mammalian serotonin$_2$-like (5-HT$_2$-like) binding sites in juvenile rainbow trout (*Oncorhynchus mykiss*) hypothalamus, were examined by radioligand binding assay using the tritiated analog of a selective serotonin antagonist, ketanserin ([^{3}H]ketanserin), as the radioligand. Specific [^{3}H]ketanserin binding (B_{sp}) to juvenile hypothalamus membrane was tissue-dependent where B_{sp} increased linearly with tissue concentration. Therefore, 1 hypothalamus-equivalent per tube (1100 $\pm$ 115 cpm/mg protein) was subsequently used throughout the rest of the first phase. In association experiments (n=5), B_{sp} increased progressively with time to achieve equilibrium binding levels (1192 $\pm$ 120 cpm/mg protein) which remained stable for at least 60 min thereafter; k_{obs}, and k_{+1} were 0.032 and 0.048 $min^{-1}nM^{-1}$, respectively. This consistent, and relatively stable association of radioligand to the binding site indicates good stability of [^{3}H]ketanserin binding to this binding site. In dissociation experiments, B_{sp} completely dissociated within 20 min following addition of excess ketanserin; k_{-1}, and $t_{1/2}$ were 0.0803 min^{-1} and 8.7min, respectively. This pattern of [^{3}H]ketanserin binding to this binding site is consistent with the association and dissociation kinetics of radioligand binding to a receptor. B_{sp} was saturable (2500 $\pm$ 256 cpm/mg protein); Scatchard-calculated values for the equilibrium dissociation constant (K_D) and capacity (B_{MAX}) were 0.48nM, and 125 fmol/mg protein, respectively, indicating the presence of a finite population of high-affinity 5-HT$_2$-like binding sites. B_{sp} was differentially displaced by various competitors, with a rank order of potency of ketanserin = mianserin > ritanserin > 5-HT = spiperone >> methiothepin mesylate > metergoline = DOI > 2-methyl-5-HT > α-methyl-5-HT >>>>5-HIAA = reserpine. This rank order suggests that specific 5-HT$_2$

agonists and antagonists displace specifically bound [^{3}H]ketanserin more effectively compared to non-specific competitors, and in a manner comparable with the 5-HT_2-like binding site of mammals. Collectively, these findings provide pharmacological evidence for the existence of a 5HT_2-like receptor subtype in the trout hypothalamus.

In the second phase of this study, the distribution of these 5-HT_2-like binding sites was compared with serotonin (5-HT) content in corresponding brain regions of juvenile (no obvious gonads present) and sexually recrudescing female (presence of ovaries) trout. Amounts of specifically bound [^{3}H]ketanserin (B_{sp}) varied widely among brain regions. Levels of B_{sp} were significantly greater in the hypothalamus than in the olfactory lobe, which were at least three-fold greater than all other tissues examined. The magnitude of B_{sp} was hypothalamus >> olfactory lobes >> optic lobes > pre-optic area >>>spinal cord >>>> pituitary. In juveniles, highest B_{sp} levels were detected in the hypothalamus (1620 $\pm$ 109 cpm/mg protein), which were larger than B_{sp} levels in the olfactory lobe (987 $\pm$ 67 cpm/mg protein), which in turn were larger than levels in the optic lobe, pre-optic area and spinal cord. Similarly, in sexually recrudescing females, highest B_{sp} levels were detected in the hypothalamus (1100 $\pm$ 127 cpm/mg protein), which were larger than B_{sp} levels in the olfactory lobe (423 $\pm$ 34 cpm/mg protein), which in turn were larger than levels in the optic lobe, pre-optic area and spinal cord. Binding site densities in the hypothalamus, olfactory lobe, pre-optic area, and optic lobes were greater in juveniles compared with corresponding tissues from sexually recrudescing females. In contrast, binding site densities in the spinal cord did not differ between juveniles and sexually recrudescing females. These results indicate possible age-related changes in the density of specific 5-HT_2-like binding sites in rainbow trout brain regions.

HPLC-EC analysis of biogenic amine standards resulted in a stereotypic elution pattern; peaks were consistently separable in time with highly conserved retention times. Concentrations of 5-HT varied widely among brain regions of juvenile and sexually recrudescing female trout. In juveniles, 5-HT concentration was three to four fold greater in the hypothalamus (228 $\pm$ 12 ng/g tissue) than in both the pre-optic area (56 $\pm$ 7 ng/g tissue) and olfactory lobe (78 $\pm$ 13 ng/g tissue). Similarly, in sexually recrudescing females, 5-HT concentration was greater in the hypothalamus (93 $\pm$ 7 ng/g) than both pre-optic area (21 $\pm$ 2 ng/g) and olfactory lobe (73 $\pm$ 20 ng/g). 5-HT concentration was significantly greater in the hypothalamus of juveniles compared with sexually recrudescing females.

In general, tissue 5-HT levels (pmoles/g tissue) were greater in the brain regions of juvenile trout, than in corresponding brain regions of sexually recrudescent females, with the exception of the olfactory lobe, where 5-HT levels were comparable between juveniles and sexually recrudescent females.

When B_{sp} density and 5-HT content are represented in units of fmol/g tissue and pmol/g tissue, respectively, it is possible to compare them with each other. The ratio of 5-HT content to specifically bound [^{3}H]ketanserin in all trout brain regions was collectively 947 ($\pm$ 240):1 with the exception of juvenile olfactory lobe where 5-HT content was approximately 300-fold greater than specifically bound [^{3}H]ketanserin. This suggests high ratio of neurotransmitter:binding sites for the serotonergic system in trout brain regions, and possibly higher neuronal activity in trout olfactory lobe compared with other brain regions.

In conclusion, the findings in these two studies collectively suggest, the existence of a

5-HT_2-like binding site in trout brain, which has variable distribution among tissue types in the same age class of fish, and relatively higher levels in corresponding brain regions of juveniles compared with sexually recrudescing females. The levels of these 5-HT_2-like binding sites were lower than levels of 5-HT detected in corresponding brain regions, however, similar neurotransmitter:binding site ratios were observed in all brain regions with the exception of the juvenile olfactory lobe. Results of this study suggest that the levels of 5-HT can be predictive of local levels of specific [^{3}H]ketanserin binding, and that 5-HT plays important age-related role (s) in rainbow trout brain regions.

Key Words: Serotonin, [^{3}H]ketanserin, High Performance Liquid Chromatography-Electrochemical Detection, and sexually recrudescing female rainbow trout.

CHAPTER 1

General Introduction

General Introduction

This research examines the existence of a specific [^{3}H]ketanserin binding site (mammalian 5-HT_2-like binding site) in trout hypothalamic membranes as well as the relationship between tissue 5-HT content and specific [^{3}H]ketanserin binding site density in trout brain regions.

5-HT: BIOSYNTHESIS AND METABOLISM

5-HT, an indoleamine neurotransmitter, was first identified in mammalian blood platelets (Rapport et al. 1948). It has since been found in the CNS of annelids (earthworm, Sloley 1994), arthropods (insects, Sloley and Orikasa 1988), as well as in the CNS of various vertebrates including fishes (Kah and Chambolle 1983, Ekstrom and Van Veen 1984, Corio et al. 1991), birds (Hall et al 1986), reptiles (Doshi et al. 1975), and mammals (Dahlstrom and Fuxe 1964; Dinan 1996).

5-HT biosynthesis and metabolism have been most extensively studied in mammals. In the pre-synaptic neuron, serotonin biosynthesis and metabolism starts with the conversion of tryptophan to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase. 5-HTP is converted to 5-HT via aromatic amino acid decarboxylase (Frazer and Hensler 1994). 5-HT molecules are stored in vesicles which lodge at activation sites in the pre-synaptic neuron terminal (Tamir and Gershan 1990). 5-HT molecules released from the pre-synaptic neuron enter the synaptic cleft, may subsequently bind specifically with post-synaptic 5-HT receptor subtypes. The specific interaction of ligand molecule (5-HT) and binding site triggers a series of secondary messages in the post-synaptic neuron. Any 5-HT remaining in the synaptic cleft may be transported back into the pre-synaptic neuron via specific uptake carrier proteins; this reabsorbed 5-HT may be metabolized to an inactive

metabolite, 5-hydroxy-indoleacetic acid (5-HIAA), through the action of monoamine oxidases (MAO's; Frazer and Hensler 1994).

5-HT PATHWAYS IN TELEOST CNS

Immunocytochemical techniques have been used in bony fish (teleosts) to examine the projection of hypothalamic neurohormonal fibers into the teleost pituitary (Fryer and Maler 1981, Peter et al. 1990, Anglade et al. 1993). These fibers are in direct association with pituitary target cells. Because teleosts lack a functional hypothlamo-hypophyseal portal system, and because of this special neuroanatomical arrangement, teleosts provide a unique experimental model to study 5-HT regulation of pituitary endocrine cells (Peter et al. 1990). 5-HT neurons exist in the midbrain, brain stem and diencephalon of various teleost species: goldfish (*Carassius auratus*; Kah and Chambolle 1983), African catfish (*Clarias gariepinus*; Corio et al. 1991) and rainbow trout (*Salmo gairdneri*; Frankenhuis-van den Heuvel and Nieuwenhuys 1984). 5-HT producing neurons are located in high concentrations in the nucleus raphe' medialis of the teleost midbrain region (Kah and Chambolle 1983, Corio et al. 1991, and Frankenhuis-van den Heuvel and Nieuwenhuys 1984). Large numbers of serotonergic neurons originating in the nucleus raphe' medialis extend fibers to various teleost brain regions. For example, neuronal fibers originating in the raphe' nucleus extend to: the hypothalamus and ventral thalamus, in three spined stickleback (Ekstrom and Van Veen 1984), the pre-optic nucleus of the African catfish (Corio et al. 1991), and the pars distalis of the pituitary gland, medulla oblongata, spinal cord, and olfactory lobe of the goldfish (Kah and Chambolle 1983). This extensive distribution of 5-HT neuronal fibers in teleost brain and pituitary regions implies a local

biological function for 5-HT as well as the existence of specific 5-HT binding sites in these regions of teleost brain.

ROLES OF 5-HT IN TELEOST CNS

Serotonin mediated regulation of neuroendocrine bioactivity in teleost brain regions has been the subject of ongoing investigations. To illustrate, in *in vitro* perifused goldfish pituitary fragments, 5-HT has an inhibitory effect on the secretion of growth hormone (GH) through an 5-HT_2 receptor subtype, and a stimulatory effect on the secretion of gonadotropin (GtH) through 5-HT_2 and possibly 5-HT_1 receptor subtypes (Wong et al. 1998). In the pituitary of both female Atlantic croaker (*Micropogonias undulatus*; Khan and Thomas 1992) and female and male goldfish (Somoza et al. 1988), 5-HT stimulates the release of maturational GtH. In the pituitaries of both male and female mollies (*Poecilia latipinna*), serotonin mildly stimulates GtH secretion at different stages of reproduction (Groves and Batten 1986). Collectively, these findings on 5-HT bioactivity in teleost pituitary imply the presence of specific 5-HT binding sites in the teleost pituitary. In the Indian catfish (*Heteropneutes fossilis*), hypothalamic serotonin levels are known to vary with annual physiological changes (Senthilkumar and Joy 1993). For example, levels of 5-HT in trout brain and pituitary regions undergo various physiological fluctuations during ovarian recrudescence and ovulation (Saligaut et al. 1992). These changes exert major effects on the trout hypothalamo-hypophysial complex, which in turn, results in changes in rainbow trout annual reproductive cycle (Saligaut et al. 1992). These findings imply the existence of specific 5-HT binding sites in these brain regions.

5-HT IN THE CNS OF MAMMALS

In mammals, serotonin cell bodies are abundant in the raphe´ nucleus of the midbrain region (Dahlstrom and Fuxe 1964; Frazer and Hensler 1994; Tork 1990). Axon fibers project from the raphe´ nucleus to various regions of the rat brain including the caudate-putamen (Jacobs et al. 1974, Stienbusch 1981), the hippocampus (Stienbusch 1981), and the paraventricular nucleus of the hypothalamus (Van de Kar 1991).

Serotonin regulates various neuroendocrine and psychological functions in mammalian CNS via its complex receptor system. Serotonin receptor subtypes in mammals are known to be structurally and pharmacologically diverse (Hoyer et al. 1984, Saudou and Hen 1994, Frazer et al 1990, Hoyer and Shoeffter 1991). These 5-HT receptors can be classified into at least three, possibly up to seven, classes of receptors (Lacau-Mengido et al. 1996). They comprise the 5-HT_1, 5-HT_2, and 5-HT_3 classes, the "uncloned" 5-HT_4 receptor and the recombinant receptors 5-ht_5, 5-ht_6 and 5-ht_7. Previous studies have investigated the roles of different serotonin receptor subtypes in neuroendocrine responses to the activation of the serotonergic system. To illustrate, 5-HT acting at the 5-HT_3 receptor mediates FSH (follicle stimulating hormone) and LH (leutinizing hormone) release in female infantile rats; by contrast, 5-HT_{2C} or $_{2A}$ receptor subtypes participate in the release of prolactin at this stage (Lacau-Mengido et al. 1996). Evidence based partly on the ability of selective serotonin receptor antagonists to prevent the increase in ACTH and corticosterone in rats *in vivo* (Fuller 1990; 1996) in humans (Dinan 1996, Van de Kar 1991), has implicated 5-HT_{1A} and 5-$HT_{2/1C}$ receptor subtypes in regulating CRF secretion. Serotonin directly regulates the release of TRH (thyrotropin releasing hormone) in human anterior pituitary (Tuomisto and Mannisto 1985) via 5-HT_{1A}

or 5-HT_{1B} receptor subtypes, oxytocin, vasopressin and renin in both humans and rats (Van de Kar and Brownfield 1993, Tuomisto and Mannisto 1985; Van de Kar 1991) stimulated by the 5-HT_2 receptor subtype. Besides regulating physiological functions in mammals 5-HT also plays the role of an important psychological modulator. For example, 5-HT abnormalities in humans are directly linked to a number of psychiatric disorders, particularly schizophrenia and depression (Kapur and Remington 1996).

OBJECTIVES OF THIS RESEARCH

Although specific 5-HT binding sites and their physiological functions have been studied extensively in mammals, there is little to no direct information on the existence or distribution of specific 5-HT binding sites in teleosts. Therefore, this research investigates the existence and pharmacological characteristics of specific 5-HT_2-like binding sites in the trout hypothalamus, and attempts to describe the distribution of these binding sites in selected brain regions of juvenile and sexually recrudescing female trout. In the first phase, I use [^{3}H]ketanserin (selective 5-HT_2 antagonist) in a radioligand-binding assay to identify specific [^{3}H]ketanserin binding sites and to describe the structural criteria for ligand recognition by these 5-HT_2-like sites. Ketanserin has been used in previous determinations of existing 5-HT_2 receptor subtypes (Leysen et al. 1982, Leysen et al. 1984, Vanhoutte et al. 1983, Janssen 1983) in mammalian CNS. In order to provide substantial evidence for the specific binding of [^{3}H]ketanserin to trout hypothalamic 5-HT_2-like binding sites various pharmacological characterization experiments (Bylund and Yamamura 1990) including, saturation analysis, kinetic (association and dissociation) analysis, and displacement analysis, using a diverse assembly of pharmacological probes are conducted.

In a second phase of this research, I examine the distribution levels of 5-HT_2-like binding sites in corresponding brain regions of juvenile and sexually recrudescing females, and investigate changes in receptor distribution in rainbow trout related to reproductive status. I use [^{3}H]ketanserin radioligand binding assay and HPLC-EC analysis to detect levels of specific 5-HT_2-like binding sites and 5-HT respectively, in the brain regions of juvenile and sexually recrudescing females. Specific [^{3}H]ketanserin binding levels are then compared to 5-HT content in corresponding brain regions as well as between juvenile and sexually recrudescing females.

This investigation provides valuable information on the existence and pharmacological characteristics of specific [^{3}H]ketanserin binding sites in selected brain regions of juvenile and sexually recrudescing female rainbow trout. Moreover, comparison of local 5-HT contents and binding site densities will permit consideration of mechanisms for their mutual regulation.

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CHAPTER 2

*Specific binding of [^{3}H]ketanserin to the hypothalamus membranes of juvenile rainbow trout (*Oncorhynchus mykiss*).*

Specific binding of [^{3}H]ketanserin to hypothalamus membranes of juvenile rainbow trout, *Oncorhynchus mykiss*

Abstract

This study examines the existence and pharmacological specificity of [^{3}H]ketanserin binding in hypothalamus of juvenile rainbow trout. Hypothalamic membranes were incubated with [^{3}H]ketanserin (selective $5HT_2$-antagonist) under several experimental conditions; reactions were terminated by filtration and bound radioactivity was counted by liquid scintillation spectroscopy. Tissue dilution experiments revealed that specific [^{3}H]ketanserin binding (B_{sp}) was tissue-dependent; 1 hypothalamus-equivalent per tube (1100 $\pm$ 115 cpm/mg protein) was subsequently used throughout the rest of this study. In association experiments, B_{sp} increased progressively with time, achieved equilibrium binding levels (1192 $\pm$ 120 cpm/mg protein) within 80 min, and remained stable for at least 60 min thereafter; k_{obs}, and k_{+1} were 0.032 and 0.048 $min^{-1}nM^{-1}$, respectively. In dissociation experiments, B_{sp} completely dissociated within 20 min following addition of excess ketanserin; k_{-1}, and $t_{1/2}$ were 0.0803 min^{-1} and 8.7min, respectively. B_{sp} was saturable (2500 $\pm$ 256 cpm/mg protein); Scatchard-calculated values for the equilibrium dissociation constant (K_D) and capacity (B_{MAX}) were 0.48nM, and 125 fmol/mg protein, respectively. B_{sp} was differentially displaced by structurally related competitors, with a rank order of potency of ketanserin = mianserin > ritanserin > 5-HT = spiperone >> methiothepin mesylate > metergoline = DOI > 2-methyl-5-HT > α-methyl-5-HT >>>>5-HIAA = reserpine. These findings provide pharmacological evidence for the presence of a $5HT_2$-like receptor subtype in the trout hypothalamus.

Key Words: hypothalamus, [^{3}H]ketanserin, 5-HT_2 receptor subtype, and rainbow trout.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT), an indoleamine neurotransmitter /neurohormone, is present in the nervous systems of many invertebrates including annelids (Sloley 1994), and insects (Sloley and Orikasa 1988, Lutz and Tyrer 1988). 5-HT is also prominent in the central nervous system (CNS) of all studied vertebrates (Brodie et al. 1964, Karki and Lahovaara 1965), including fish (Kah and Chambolle 1983, Ekstrom and Van Veen 1984, Corio et al. 1991), birds (Hall et al. 1986), reptiles (Doshi et al. 1975), and mammals (Dahlstrom and Fuxe 1964; Dinan 1996).

Immunocytochemical studies of brain regions of teleosts (bony fishes) have demonstrated populations of 5-HT neurons in the midbrain, brain stem and diencephalon of goldfish (*Carassius auratus*; Kah and Chambolle 1983), African catfish (*Clarias gariepinus*; Corio et al. 1991), and rainbow trout (*Salmo gairdneri*; Frankenhuis-van den Heuvel and Nieuwenhuys 1984). Particularly high densities of 5-HT have been found in the nucleus raphe' medialis in various species of teleosts including the African catfish, Corio et al. 1991), three spined stickleback (*Gasterosteus aculeatus* L; Ekstrom and Van Veen 1984), and sockeye salmon (*Oncorhynchus nerka* Walbaum; Ekstrom and Ebbesson 1989). The raphe' nucleus projects large numbers of serotonergic axons to multiple regions of the teleost brain, including the hypothalamus, ventral thalamus, and pituitary of the three spined stickleback (Ekstrom and Van Veen 1984), pars distalis of the pituitary gland, medulla oblongata, olfactory lobes, pre-optic area optic lobe and spinal cord of goldfish (Kah and Chambolle 1983), as well as the pre-optic nucleus in the African catfish (Corio et al. 1991). A few scattered 5-HT-varicosities have also been observed in the cerebellum of the three spined stickleback (Ekstrom and Van Veen 1984). Serotonergic

neurons also occur in the pineal and circumventricular areas of rainbow trout (Hafeez and Zerihun 1976). This distribution of 5-HT is consistent with aspects of 5-HT bioactivity in the teleost brain: pituitary axis.

Teleost pituitary function and hormone secretion is directly and indirectly regulated by serotonergic input. For example, 5-HT regulates the secretion of growth hormone (GH) and gonadotropin (GtH) (Somoza and Peter 1991) from *in vitro* perifused goldfish pituitary fragments; 5-HT also stimulates release of maturational GtH from the pituitary of female Atlantic croaker (*Micropogonias undulatus*; Khan and Thomas 1992) and female and male goldfish (Somoza et al. 1988). In rainbow trout, Saligaut et al. (1992) demonstrated physiological fluctuations in hypothalamus and pituitary serotonin levels during ovarian recrudescence and ovulation. Senthilkumar and Joy (1993) also observed similar annual variations of serotonin levels in the hypothalamus of the Indian catfish (*Heteropneutes fossilis*). These findings implicate 5-HT as a major neuroendocrine regulator in teleosts. This evidence for the presence of 5-HT pathways and bioactivity in teleost brain regions, logically suggests for the presence of 5-HT binding sites (receptors) in these brain regions. This study was conducted to determine the existence of any such binding sites.

In mammals, 5-HT-receptors are classified into 7 major receptor subtypes (for reviews see: Sanders-Bush and Mayer 1996, Alexander and Peters 1997, Watson and Gridlestone 1995). These receptors, acting through intracellular signaling systems, regulate, in part, the mammalian CNS, including brain: pituitary axes (Pandey et al 1994, Rahimian and Hrdina 1995, Conn and Sanders-Bush 1987). In mammals 5-HT exerts its influence in the brain: pituitary axis primarily through activation of the 5-HT_2 receptor

subtype (Dinan 1996, Fuller 1992, Leysen and Pauwels 1990). By contrast, there is little to no direct information on 5-HT receptors in teleosts.

Previous studies in goldfish (Wong et al. 1998) have already suggested that 5-HT has an inhibitory effect on the secretion of growth hormone (GH) through an 5-HT_2 receptor subtype, and a stimulatory effect on the secretion of gonadotropin (GtH) through 5-HT_2 and possibly 5-HT_1 receptor subtypes. Ketanserin, a selective 5-HT_2 antagonist, has been used to identify the 5-HT_2 receptor subtype in mammal CNS models (Sanders-Bush and Mayer 1996, Wolf and Shutz 1997, Mokler et al 1997, Marazziti et al 1997, Janssen 1983, Leysen et al 1981. The purpose of this study is to evaluate the existence and binding characteristics of [^{3}H]ketanserin to hypothalamus membranes of juvenile rainbow trout.

Materials and methods

Experimental animals

Fingerling rainbow trout (*Oncorhynchus mykiss*; Rainbow Springs Trout Hatchery, Thamesford, Ont.) were raised to juveniles in the Lakehead University Aquatic Animal Research facility in flow-through aquaria with dechlorinated water at simulated ambient temperature (annual range, 5 to 16^0C) and photoperiod (annual range, 8 to 14h photophase). Fish were fed commercial trout pellets daily (1 to 3% body weight; Zeigler trout feed, Thunder Bay Co-Op. Juvenile fish (38 $\pm$ 8 cm long) were approximately 24 months old and did not possess obvious gonads; any fish with the obvious presence of gonads were excluded from this study. All fish were maintained and handled in accordance with guidelines established by the Canadian Council on Animal Care as well as the Ontario Animals for Research Act. In all cases, fish were anesthetized with tricaine methanesulphonate (MS-222, 0.5g/liter; Syndel Laboratories, Vancouver, B.C.) prior to any handling, then killed by spinal transection posterior to the medulla oblongata.

Tissue preparation for binding assay

Whole brains were isolated and placed in ice-cold assay buffer (50 mM Tris-HCl, pH = 7.4). Each hypothalamus, the region below the thalamus and posterior to the telencephalon, commencing at the optic tract and extending posteriorly to the nucleus diffusus lobi inferioris (Billiard and Peter 1982), was surgically isolated using recurved dissection sissors. For each independent experiment, hypothalami were harvested and pooled into Corning 15-ml polystyrene centrifuge tubes, suspended in liquid N_2, (preliminary experiments showed no obvious difference in [^{3}H]ketanserin binding to fresh or frozen rainbow trout hypothalamus preparations, Agrawal and Omeljaniuk,

unpublished). Frozen hypothalami were stored in liquid N_2 until the following day for inclusion in a radioligand binding assay.

[^{3}H]Ketanserin binding assay

Frozen tissue was transferred to an ice-cold glass mortar and combined with (100 µl/original tissue sample) ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4; 0.32 M sucrose; Gallaher and Wang 1990; Leysen et al. 1982); all subsequent procedures were carried out at 0 to 4^{0}C. Tissue was homogenized with ten strokes of a motor-driven Potter-Elvehjem homogenizer (0.125-mm clearance). Homogenates were transferred to 10-ml polypropylene centrifuge tubes and centrifuged at 1000 x g for 20min, the resulting supernatants were subsequently aspirated and transferred to Beckman Ultra-Clear centrifuge tubes (13 x 32mm) and centrifuged at 100,000 x g for 30min. The resulting supernatants were discarded and pellets were homogenized in 100 µl assay buffer per original tissue sample, and centrifuged at 100,000g (30min). To prepare the membrane suspension, the resulting supernatant was decanted to waste and pellet suspended in 100µl assay buffer per original tissue sample.

Typically, a 100µl aliquot of membrane suspension was incubated with 100 µl [^{3}H] ketanserin (NEN-Dupont, Boston, MA; 66.4 Ci/mmol) and either 100µl assay buffer, to determine total binding (B_o), or 100µl unlabelled ketanserin (10µM) to estimate non-specific binding (NSB), resulting in a final volume of 300µl. Specific binding (B_{sp}) was calculated as the difference between total (B_o) and nonspecific binding (NSB). Binding reactions were terminated by filtration through Whatman GF/B filters, presoaked overnight in assay buffer containing 0.3% polyethyleneimine to reduce nonspecific binding (Schwartzentruber and Omeljaniuk 1994), followed by 3 rinses of 3 ml ice-cold assay

buffer. Filters placed in 6-ml scintillation vials (Beckman, Mississauga, ON) were incubated overnight in 4 ml of scintillation cocktail (Readysafe™; Beckman, Mississauga, ON) and radioactivity was determined by liquid scintillation spectroscopy at 50% counting efficiency.

Protein Determination

Protein content was determined by the Bradford method (Bradford 1976) using Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, CA) and bovine serum albumin (Sigma Chemicals, St. Louis, MO) as a protein standard.

Data Analysis

Specific binding (B_{sp}) was calculated as the difference between mean total (B_o) and mean nonspecific (NSB); the standard error of mean B_{sp} (B_{sp}SEM) was calculated as (B_oSEM ^{2} + NSBSEM ^{2})$^{1/2}$ (Hulme and Birdsall 1992).

Where indicated, kinetic data (association and dissociation) were transformed based on the method of Bylund and Yamamura (1990) to determine observed rate of association (k_{obs}), association rate constant (k_{+1}), and dissociation rate constant (k_{-1}), and to calculate the kinetically derived dissociation constant (k_{-1}/k_{+1}). For association experiments, k_{obs} was calculated from the plot of ln [B_e /B_e-B_{sp}] versus time (min), where B_e is the level of binding at equilibrium and B_{sp} is specific binding at each time interval; k_{obs} is slope of the straight line derived from the linear regression equation. From dissociation analysis, k_{-1} was calculated from the linear regression analysis of lnB_{sp}/B_{zerot} versus time (min), where B_{sp} is specific binding at each time interval and B_{zerot} is specific binding just prior to the addition of excess unlabelled ketanserin, and k_{-1} is the slope of the line derived from linear regression. The association rate constant (k_{+1}) was calculated as

$k_{obs} - k_{-1} / F$, where F is the concentration of free [^{3}H]ketanserin (nM).

Data from equilibrium binding experiments were used to calculate the half-maximal inhibitory concentration (IC_{50}), maximum number of receptors bound by radioligand (B_{MAX}), and equilibrium dissociation constant (K_i). Scatchard analysis (1949) of triplicate independent experiments was used to calculate K_D (K_i) and B_{MAX} from the data in saturation analysis as well as competitive displacement analysis of [^{3}H]ketanserin binding; results in each were reported as (mean $\pm$ SEM). K_i values were comparable to those calculated according to Cheng & Prusoff (1973), $K_i = IC_{50} / [1 + C / K_D]$, where C is concentration of radioligand and K_D is dissociation rate constant obtained from saturation experiments. Half-maximal inhibitory concentration (IC_{50}) values for each competitor were estimated from logit-log plots by plotting logit (logit = ln[P/(100 –P)], P is percent bound) of total [^{3}H]ketanserin binding to trout hypothalamic membrane preparation versus -log [competitor, M]. The IC_{50} is 50% binding, and the logit of 50 % [ln (1)] is 0. Thus, the IC_{50} was determined by linear correlation (Bylund and Yamamura 1990) (not shown). LIGAND analysis (Munson and Rodbard 1980) of displacement data confirmed results of Scatchard analyses (Scatchard 1949).

Results

Effect of tissue concentration on [^{3}H]ketanserin binding

In five independent experiments, various amounts of rainbow trout hypothalamus membrane preparation were incubated in triplicate with [^{3}H]ketanserin for 90 minutes prior to termination. Specifically bound [^{3}H]ketanserin increased linearly with protein concentration between 0.07 and 0.42 mg protein, with a relationship of B_{sp} (cpm) = 1539 protein (mg) + 9.42 (r^2 = 0.94) (Fig. 1). In these experiments, B_o and B_{sp} for one hypothalamus-equivalent per tube (0.17 $\pm$ 0.03 mg protein) were 2161 $\pm$ 260 and 1100 $\pm$ 115 cpm, respectively. NSB increased with protein and generally represented 38.4% of B_o. Based on these results, one hypothalamus-equivalent per tube was used in subsequent experiments.

Association of [^{3}H]ketanserin.

In five independent experiments, one hypothalamus-equivalent per tube was incubated in triplicate with [^{3}H]ketanserin for various time intervals prior to termination. Specific binding increased with time and reached equilibrium binding (1192 $\pm$ 120 cpm/mg protein) within 80 minutes (Fig. 2 panel A); equilibrium B_{sp} remained relatively stable for at least 60 minutes. Data were transformed (ln [B_e /(B_e-B_{sp})]), according to Bylund and Yamamura (1990), pooled and replotted as a function of time (min) (Fig. 2 panel A, inset). Linear regression analysis of this relationship (ln [B_e /(B_e-B_{sp})] = 0.032 min + 0.044, r^2=0.93) estimated k_{obs} (slope of the line) as 0.032 min; the association rate constant (k_{+1}) was subsequently estimated as 0.048 $min^{-1}nM^{-1}$

Dissociation of [^{3}H]ketanserin.

In five independent experiments, hypothalamus membrane preparation (one hypothalamus-equivalent per tube) was incubated in triplicate with [^{3}H]ketanserin for 90 minutes prior to the addition of 5000-fold excess radiostable (unlabelled) ketanserin in all the tubes. Tubes were then incubated at various time intervals before termination. B_{sp} at equilibrium (1400 $\pm$ 120 cpm/mg protein) rapidly dissociated in response to excess competitor and reached a state of complete dissociation within 20 minutes (Fig 2 panel B). Data were transformed (ln B/B_{zero}), according to Bylund and Yamamura (1990), pooled and replotted as a function of time (min) (Fig. 2 panel B, inset). Linear regression analysis of this relationship (ln B/B_{zerot} = 0.0803 min + 0.417, r^2 = 0.9) provided estimates of half-life and dissociation rate constants of $t_{1/2}$ = 8.7 min and k_{-1} =0.0803 min^{-1}, respectively. The kinetically derived dissociation constant (k_{-1}/k_{+1}) was estimated to be 1.67 nM.

Saturation analysis of [^{3}H]ketanserin.

In five independent experiments, one hypothalamus-equivalent per tube was incubated with varying concentrations of [^{3}H]ketanserin in triplicate for 90 minutes prior to termination. B_{sp} increased steadily with increasing concentrations of radioligand (between 0.25 and 4.64nM) to reach saturation levels of 2500 $\pm$ 256 cpm/mg protein (Fig. 3). Data from 6 independent experiments were pooled and analyzed by Scatchard analysis (Fig. 3, inset, Scatchard, 1949) to yield estimates of the equilibrium dissociation constant (K_D = 0.48 nM) and maximum number of binding sites (B_{MAX} 125 fmol/mg protein). The equation of the relationship was B/F = -2.07 x B

+0.03 ($r^2 = 0.95$).

Competitive displacement of [^{3}H]ketanserin

Varying concentrations of competitors were incubated with [^{3}H]ketanserin and one hypothalamus-equivalent of membrane preparation per tube for 90 minutes prior to termination. Experiments were conducted in triplicate with 3 replicate determinations per experiment. Varying competitors (Fig. 4) specifically and differentially displaced [^{3}H]ketanserin equilibrium bound to trout hypothalamus membrane preparation. Data from triplicate independent experiments for each competitor were pooled and analyzed by the method of Scatchard (1949) to determine the equilibrium dissociation constant K_D (K_i) and maximum binding capacity (B_{MAX}) for each competitor (Table 1). K_i values were found to be comparable with calculated values according to Cheng & Prusoff (1973), $K_i = IC_{50} / [1 + C / K_D]$, where C is concentration of radioligand and K_D is equilibrium dissociation constant obtained from saturation experiment. The estimated half-maximal inhibitor concentrations (IC_{50}) (Table 1) were derived from logit-log plots by plotting logit of total [^{3}H]ketanserin binding to trout hypothalamic membrane preparation versus -log [competitor, M] (Bylund and Yamamura 1990) (not shown). LIGAND analysis of displacement data indicated only a single class of binding sites, and LIGAND-derived parameter estimates were comparable with Scatchard-derived parameters. Competitors represented 5-HT receptor-antagonists (ketanserin, mianserin, ritanserin, metergoline, and methiothepin mesylate), 5-HT receptor-agonists (DOI, 2-methyl-5-HT maleate, α-methyl-5-HT maleate), a D_2/5-HT_2 antagonist (spiperone), a 5-HT metabolite (5-HIAA), and a 5-HT storage vesicle depletor (reserpine).

Comparison of binding affinity and specificity of the various competitors (Fig. 4, Table 1) reveals a rank order of potency of ketanserin = mianserin > ritanserin > 5-HT = spiperone >> methiothepin mesylate > metergoline = DOI > 2-methyl-5-HT > α-methyl-5-HT >>>>5-HIAA = reserpine.

Discussion

Our research on [^{3}H]ketanserin binding to trout hypothalamus membrane preparation indicates the presence of a single class of high affinity, low capacity sites, with binding specificity reminiscent of the mammalian 5-HT_2 receptor family. Radioligand binding is an effective tool in 5-HT receptor studies (Gallaher and Wang 1990, Hamon 1984, Leysen et al. 1981; 1982, Hulme and Birdsall 1991). We used [^{3}H]ketanserin, a 5-HT_2 receptor antagonist (Leysen et al. 1981, 1982), in our examination of rainbow trout hypothalamus as ketanserin has been employed in many mammalian studies of 5-HT_2 receptor binding (Leysen et al 1981; 1982, Leysen and Pauwels 1990). [^{3}H]Ketanserin is particularly useful as a 5-HT_2 radioligand since it has no prominent antagonistic or agonistic (Janssen 1983) activity on other 5-HT receptor subtypes besides 5-HT_2. Leysen et al. (1981), however, demonstrated one exception to this binding specificity, in rat prefrontal cortex, where ketanserin exhibited small and atypical crossreactivity with α1-sites (5 times less potent at this receptor than at the 5-HT_2 binding site), H1-sites (5 times lower binding affinity for this receptor than for 5-HT_2 binding site), and DA:D_2-sites (100 times weaker affinity for this receptor than for 5-HT_2 binding sites). Because of its marked selectivity for 5-HT_2 receptors and high potency in mammalian brain, ketanserin is the most suitable and available pharmacological probe for our research on teleost brain membrane preparations.

[^{3}H]Ketanserin binding to hypothalamic membrane preparation was saturable, thereby defining a finite number of binding sites (Fig 3). Scatchard analysis (Scatchard 1949) estimated the affinity (K_D) and capacity (B_{MAX}) of the sites as 0.48 nM and 125 fmol/mg protein, respectively. Our findings on the affinity of the juvenile trout hypothalamus [^{3}H]ketanserin binding site are comparable to those previously reported on

rat prefrontal cortex (K_D = 0.42 ± 0.02 nM, Leysen et al. 1982; Leysen and Pauwels 1990). However, the density of 5-HT_2 binding sites in trout hypothalamus is somewhat greater than that of the rat pre-frontal cortex (B_{MAX} = 33.1± 1.2 fmol/mg protein, Leysen et al. 1982, Leysen and Pauwels 1990).

Specific binding of [^{3}H]ketanserin incubated with trout hypothalamus membrane preparation reached equilibrium within 80 min and remained bound for at least 60 minutes thereafter (Fig. 2 panel A), with an association rate constant of k_{+1} = 0.048 $min^{-1}nM^{-1}$. The rate of association in teleost hypothalamus was slower than that observed in the rat prefrontal cortex, which according to the authors was too fast to be measured accurately (Leysen et al. 1982). This difference in speed of binding may be due to their higher incubation temperature (37°C, mammalian body) compared with our incubation temperature of 4°C (average trout body temperature range is 0 to 16^{0}C); as well, the lower incubation temperature used in our experiments may suppress ligand dissociation (Bylund and Yamamura 1990) and receptor degradation thus accounting in part for our larger observed B_{MAX}.

B_{sp} at equilibrium dissociated rapidly, to completely dissociate within 20 min (Fig. 2 Panel B), with a dissociation rate constant of k_{-1} = 0.0803 min^{-1}, and half-life of bound radioligand receptor complex of $t_{1/2}$ = 8.7 min. The kinetically derived dissociation constant was calculated as 1.67 nM. Both k_{-1} and $t_{1/2}$ are comparable with values observed in rat prefrontal cortex (Leysen et al. 1982). However [^{3}H]ketanserin and the 5-HT_2 receptor complex in rat prefrontal cortex dissociated more quickly (k_{-1}= 0.7 min^{-1}, $t_{1/2}$ = 1 min (Leysen et al 1982). Lower temperatures are known to retard binding kinetics (Bylund and Yamamura 1990), thus explaining, in part, the lower association and

dissociation rate of [^{3}H]ketanserin binding in our trout model compared with mammals. Thus, results from association/dissociation experiments in teleost hypothalamus are comparable to [^{3}H]ketanserin binding to 5-HT_2 receptors in mammalian prefrontal cortex.

Mammalian 5-HT receptors are classified into numerous subtypes: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_4, 5-HT_{5A}, 5-HT_{5B}, 5-HT_6, and 5-HT_7 (Sanders-Bush and Mayer 1996, Alexander and Peters 1997, Watson and Gridlestone 1995, Van de Kar 1991, Hoyer et al. 1984). 5-HT agonists and antagonists bind to these receptors with variable degrees of affinity and capacity. Effective displacement of [^{3}H] ketanserin by specific competitors in the present study was as follows: ketanserin = mianserin > ritanserin > 5-HT = spiperone >> methiothepin mesylate > metergoline = DOI > 2-methyl-5-HT > α-methyl-5-HT >>>>5-HIAA = reserpine (Table 1, Fig. 4). High K_i values for a competitor suggests low binding affinity of that competitor to the receptor. Ketanserin, well known as a selective mammalian 5-HT_2 receptor-antagonist (Leysen et al 1982, Van Nueten et al. 1981, Vanhoutte et al. 1983, Leysen et al. 1984, Janssen 1983), displaces [^{3}H]ketanserin bound to teleost hypothalamus most effectively (Fig. 4) (K_i = 1.9 nM, Table 1). Mianserin, also a mammalian 5-HT receptor-antagonist, non-specifically binds to all 5-HT receptor subtypes with equal binding affinity (Leysen et al. 1982, Peroutka and Snyder 1981), and displaces bound [^{3}H]ketanserin from trout hypothalamus as effectively as ketanserin (Fig. 4). Spiperone, a DA:D_2/5-HT_2 antagonist, exhibits cross-reactivity with DA/D_2 as well as 5-HT_2 sites in a tissue-specific manner. In the present study, spiperone also displaces specifically bound [^{3}H]ketanserin from trout hypothalamus membrane preparation, suggesting possible crossreactivity of the trout hypothalamus 5-HT_2-like receptor for ketanserin and spiperone. 5-HT-antagonists

(metergoline and methiothepin mesylate) very sparingly displace [^{3}H]ketanserin bound to trout hypothalamus membrane preparation, and, if at all, only at extremely high concentrations. Similarly 5-HT-agonists (DOI, 2-methyl-5-HT, α-methyl-5-HT) have high K_i values (Table 1), implying low binding affinity to the 5-HT binding site in trout hypothalamus. Reserpine (biogenic amine depletor) and 5-HIAA (5-HT metabolite) did not displace [^{3}H]ketanserin bound to trout hypothalamus even at large concentrations (10 μM, Fig. 4). These data indicate that primarily ligands which are structurally related to ketanserin (4-substituted piperidine derivatives, 3-{2-[4 -4 fluorobenzoyl)-1-piperidinyl]ethyl-2,4 (1H, 3H)-quinazolinedione, Janssen 1983) or spiperone (8-[4 (4 fluorophenyl) 4-oxobutyl]-1-phenyl-1,3,8-triazaspirol [4,5]decan-4, Research Biochemicals International 1995) can successfully interact with the trout hypothalamic 5-HT_2-like binding site and implies a high degree of conservation of ligand recognition properties of this site. The present results support the concept that specific binding of [^{3}H]ketanserin identifies the existence of a specific 5-HT_2-like receptor in the juvenile trout hypothalamus.

Our findings also support a biological role for 5-HT as an important neuroendocrine regulator in teleosts. To illustrate, previous studies have indicated various regulatory roles for 5-HT in the secretion of gonadotropin (GtH) in teleost brain:pituitary axis (Khan and Thomas 1992, Somoza et al. 1988, Groves and Batten 1985, and Somoza and Peter 1991), as well as growth hormone (GH, Somoza and Peter (1991). Saligaut et al. (1992) demonstrated physiological fluctuations in 5-HT levels in pituitary fragments during ovarian recrudescence and ovulation of rainbow trout; Senthilkumar and Joy (1993) also observed similar annual variations of 5-HT levels in the hypothalamus of the

Indian catfish (*Heteropneutes fossilis*). Collectively, these data on the presence and dynamics of neuronal serotonergic activity, combined with our findings on the presence of a 5-HT_2-like binding site strongly imply a biological role for 5-HT in this region of the teleost brain, with potential significant involvement in reproduction.

In conclusion, we present the first direct evidence for the existence of a specific 5-HT_2-like receptor subtype in the hypothalamus of a teleost fish. [^{3}H]Ketanserin specifically bound to hypothalamic membrane preparations in a classical, one-site receptor model. Binding was tissue dependent (Fig. 1), associable (Fig. 2 panel A) reversible (Fig. 2 Panel B), as well as saturable (Fig. 3). [^{3}H]Ketanserin binding was of high affinity (nM) and low capacity (fmol/mg protein) and, notably, displaced by competitors (Fig. 4). To the best of our knowledge, these are the first findings to directly indicate the presence, and determine the pharmacological specificity of a 5-HT_2-like binding site (receptor) in the CNS of a teleost. This teleost 5-HT hypothalamic receptor may regulate various neuroendocrine functions in this region of the teleost brain and is implicated in regulation of the brain:pituitary axis.

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Fig. 1. Specific binding of [^{3}H]ketanserin to juvenile trout hypothalamus membrane preparation. [^{3}H]Ketanserin was incubated with varying dilution's of membrane preparation for 90 minutes in the absence (B_o) and presence (NSB) of unlabelled ketanserin (10 μM) at 0-4°C, prior to termination. Specifically bound [^{3}H]ketanserin (B_{sp}) was calculated as the difference between mean B_o and NSB; B_{sp} SEM was calculated as $(B_o \text{ SEM}^2 + \text{NSB SEM}^2)^{1/2}$. Linear regression analysis of colinear data (B_{sp} (cpm) = 1539 protein (mg) + 9.42, $r^2 = 0.94$) indicates a strong linear relationship between binding and protein content between 0.07 and 0.42 mg of protein. Values are means from replicate determinations (± SEM, n=4) from multiple (n=5) independent experiments.

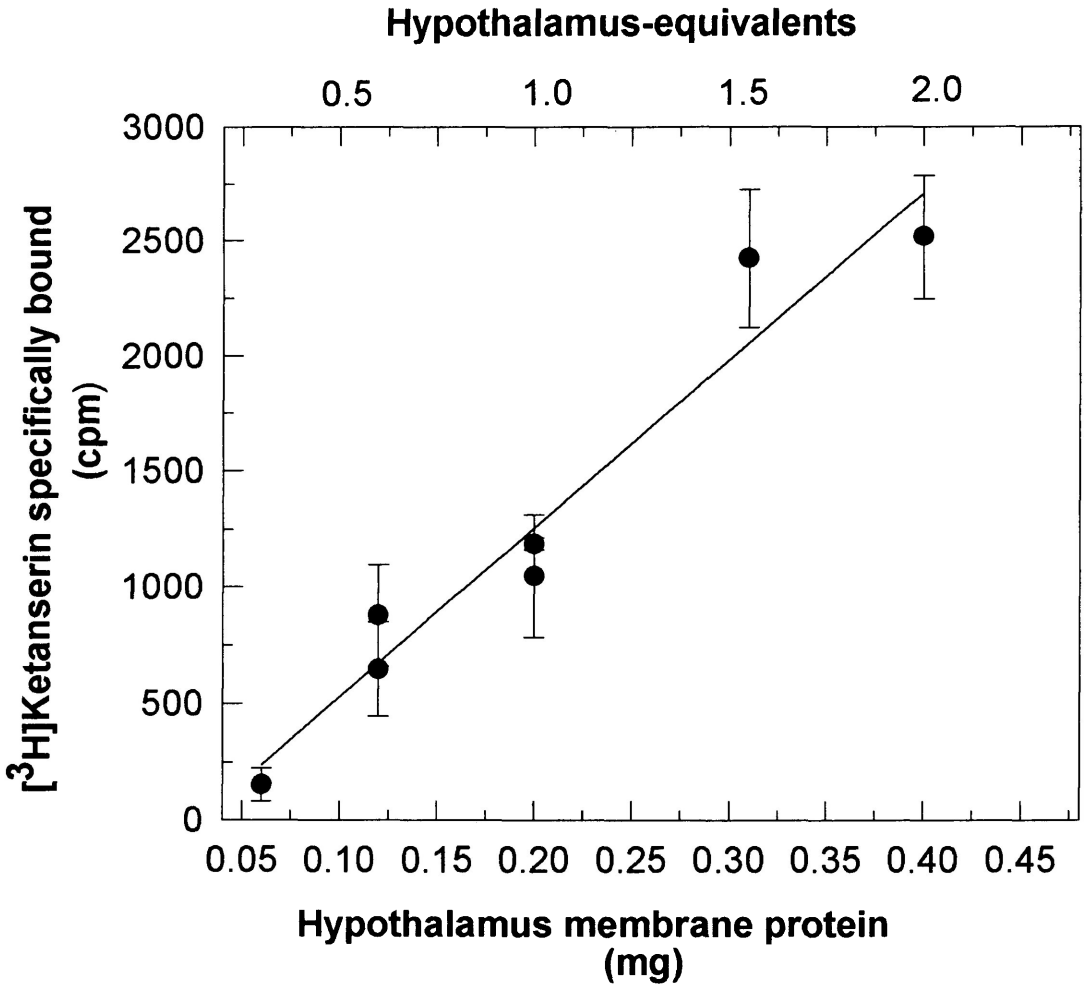
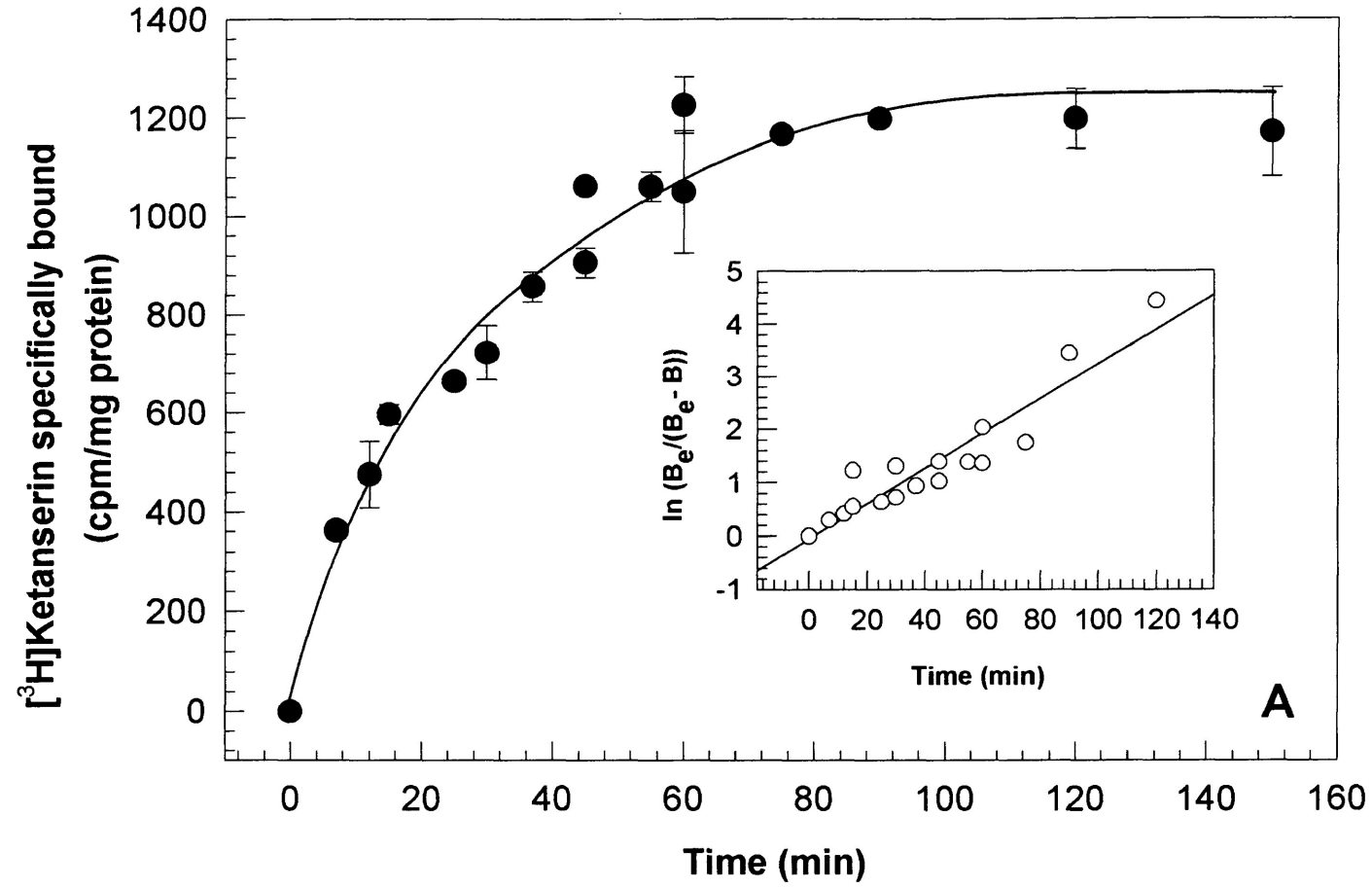

Hypothalamus-equivalents
0.5
1.0
1.5
2.0
3000
2500
2000
1500
1000
500
0
[3H]Ketanserin specifically bound (cpm)
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
Hypothalamus membrane protein (mg)

Fig. 2 Panel A. Specific binding of [^{3}H]ketanserin to juvenile trout hypothalamus membrane preparation (cpm/mg protein) as a function of time (min). Membrane suspension (1 hypothalamus-equivalent per tube) was incubated with [^{3}H]ketanserin at various time intervals in the absence (B_o) or presence (NSB) of unlabelled ketanserin (10 μM) prior to termination. Specifically bound [^{3}H]ketanserin (B_{sp}) was calculated as the difference between mean B_o and NSB, and B_{sp} SEM was calculated as $(B_oSEM^2 + NSB\ SEM^2)^{1/2}$ Values are means from replicate determinations (± SEM, n=3) from multiple (n=5) independent experiments and are represented by a common data symbol for clarity. Plot is an estimated line of best fit. Inset: Pseudo first-order association plot (Bylund and Yamamura, 1990), the slope of which (k_{obs}) is 0.032 min^{-1} with an association rate constant (k_{+1}) of 0.048 $min^{-1}nM^{-1}$ based on the equation of the line ln(Be/Be-B) = 0.032 x time + 0.0437 (r^2=0.9). **Panel B.** Dissociation of specifically bound [^{3}H]ketanserin (cpm/mg protein) from juvenile trout hypothalamus membrane preparation as a function of time (min). Membrane suspension (1 hypothalamus-equivalent per tube) was incubated with [^{3}H]ketanserin for 90 minutes in the absence (B_o) or presence (NSB) of unlabelled ketanserin (10 μM). 5000 fold excess unlabelled ketanserin was then (t=0) added to all tubes and reactions terminated at various times thereafter. Specifically bound [^{3}H]ketanserin (B_{sp}) was calculated as the difference between mean B_o and NSB, while B_{sp} SEM was calculated as $(B_oSEM^2 + NSB\ SEM^2)^{1/2}$ Values are means from replicate determinations (± SEM, n=3) from multiple (n=5) independent experiments; common symbols are used to represent data points for clarity. Inset: Semilogarithmic plot of dissociation data with $\ln(B_{sp}/B_{zerot})$ plotted as a function of time (Bylund

and Yamamura, 1990). The equation of the line is

ln (B/B_{zerot})= 0.0803 x time + 0.417 , with r^2 = 0.93. B_{zerot} is B_{sp} immediately before addition of 5000 fold excess unlabelled ketanserin. The slope of the line (k_{-1}) is 0.0803 min^{-1} with half -life ($t_{1/2}$) = ln (0.5) /k_{-1} = 8.7 min and kinetically derived dissociation constant is calculated as k_{-1}/k_{+1}= 1.67 x 10^{-9} M.


[3H]Ketanserin specifically bound (cpm/mg protein)
Time (min)
ln (Be/(Be- B))
Time (min)
A

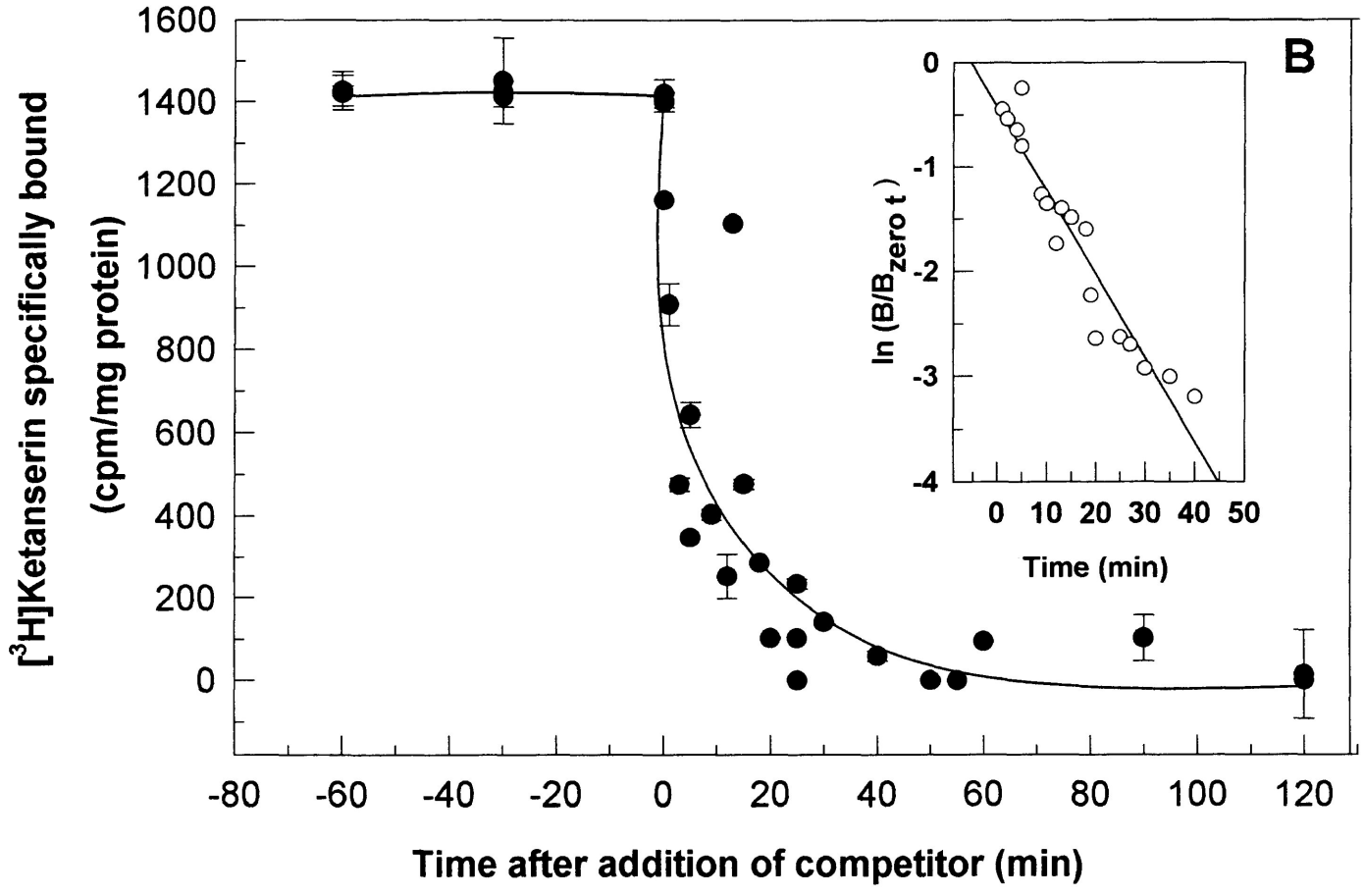

[3H]Ketanserin specifically bound (cpm/mg protein)
Time after addition of competitor (min)
ln (B/Bzero t)
Time (min)
B

Fig. 3. Saturation analysis of [^{3}H]ketanserin binding (cpm/mg protein) to juvenile trout hypothalamus membrane preparation. Membrane suspension (1 hypothalamus-equivalent per tube) was incubated with varying concentrations of [^{3}H]ketanserin for 90 minutes in the absence (B_o) and presence (NSB) of unlabelled ketanserin (10 μM) prior to termination. Specifically bound [^{3}H]ketanserin (B_{sp}) was calculated as the difference between mean B_o and NSB; B_{sp} SEM was calculated as $(B_oSEM^2 + NSB\ SEM^2)^{1/2}$. Values are means from replicate determinations (± SEM, n=3) from multiple (n=5) independent experiments; common symbols are used to represent data points for clarity. Inset: Scatchard analysis of data, regression analysis of which (Bound/Free = -2.07 x Bound (cpm/mg protein) + 0.03, r^2=0.95), was used to estimate K_D (0.48 nM) and B_{MAX} (125 fmol/mg protein).

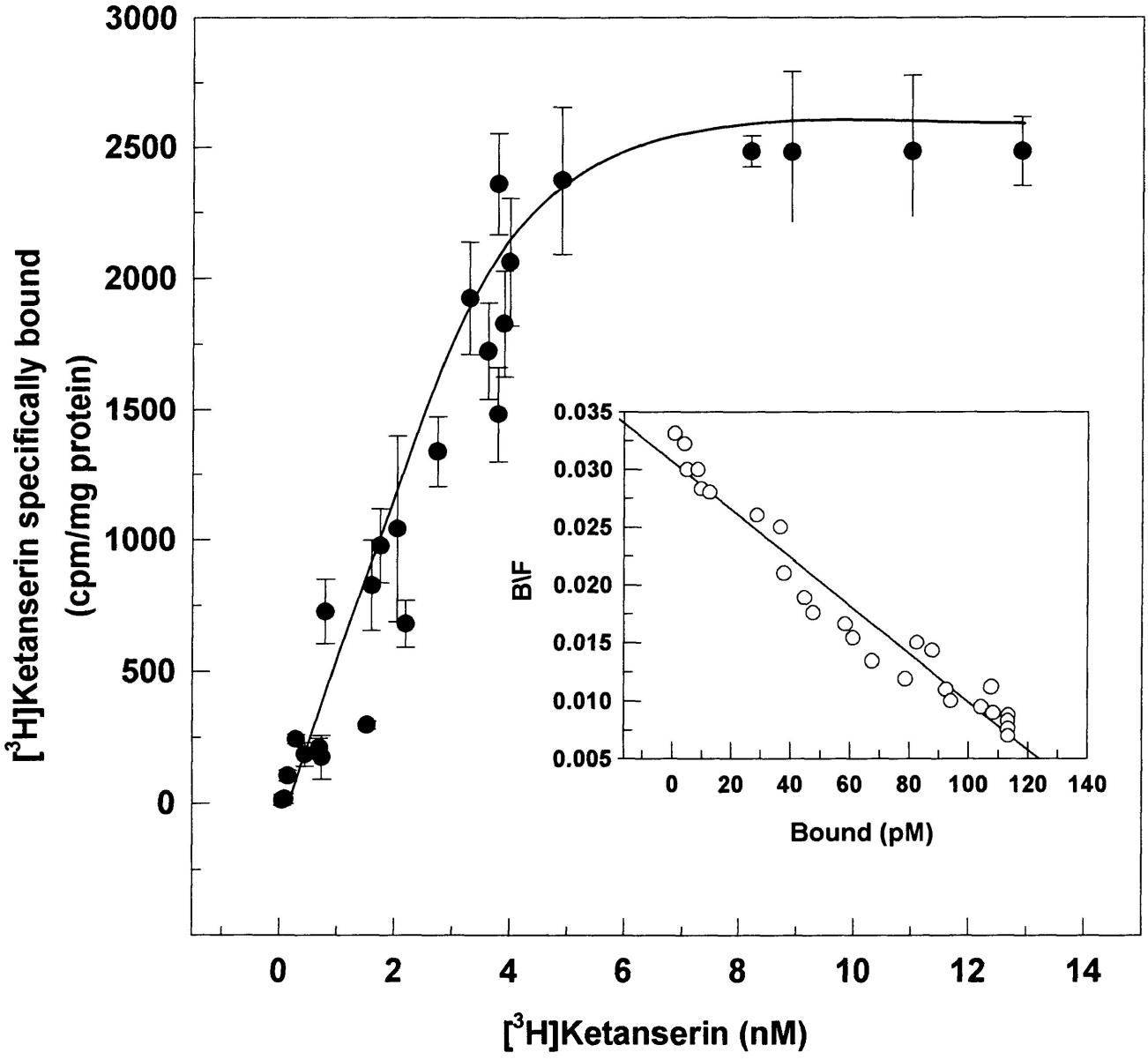

3000
2500
2000
1500
1000
500
0
[3H]Ketanserin specifically bound
(cpm/mg protein)
0
2
4
6
8
10
12
14
[3H]Ketanserin (nM)
0.035
0.030
0.025
0.020
0.015
0.010
0.005
B\F
0
20
40
60
80
100
120
140
Bound (pM)

Fig. 4. Inhibition of [^{3}H]ketanserin binding to trout hypothalamic membrane preparation by various classes of structurally related competitors. Membrane suspension (1 hypothalamus equivalent per tube) was incubated with [^{3}H]ketanserin in the absence (B_o) or presence (NSB) of various structurally related competitors. Specifically bound (B_{sp}, cpm/mg protein) [^{3}H]ketanserin was calculated as the difference between B_o and NSB, and B_{sp} SEM was calculated as (B_o SEM ^{2} + NSB SEM ^{2})$^{1/2}$. Values are means from replicate determinations ($\pm$ SEM, n=3) from multiple (n=5) independent experiments. The first panel represents displacement curves for serotonin (neurotransmitter), ketanserin (5-HT_2 antagonist), mianserin (non-specific 5-HT antagonist), and ritanserin (5-$HT_{2/1C}$ antagonist). the second panel represents displacement curves for 5-HT_1 antagonists, (metergoline and methiothepin mesylate) and spiperone (D_2/5-HT_2 antagonist), the third panel represents displacement curves for DOI (5-$HT_{2/1C}$ agonist), 2-Methyl-5-HT maleate (5-HT_3 agonist) and α-Methyl-5-HT maleate (5-HT_2 agonist), and the last panel represents displacement curves for 5-HIAA (5-HT metabolite) and reserpine (biogenic amine depletor).

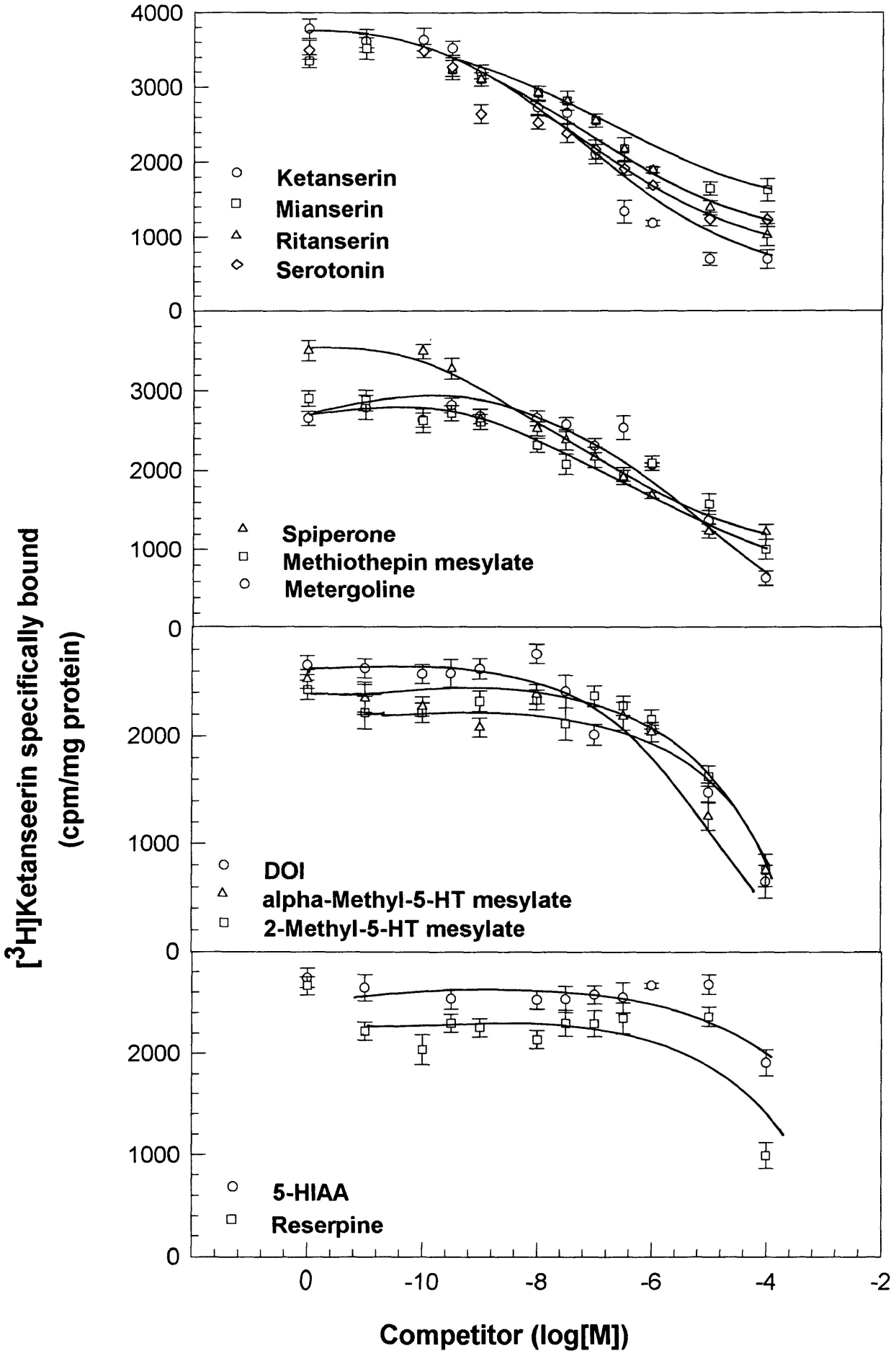

[3H]Ketanserin specifically bound (cpm/mg protein)
4000
3000
2000
1000
0
Ketanserin
Mianserin
Ritanserin
Serotonin
Spiperone
Methiothepin mesylate
Metergoline
DOI
alpha-Methyl-5-HT mesylate
2-Methyl-5-HT mesylate
5-HIAA
Reserpine
0
-10
-8
-6
-4
-2
Competitor (log[M])

Table 1. Binding constants of selected competitors for [^{3}H]ketanserin binding to trout hypothalamus membrane preparation.

COMPETITOR		K_i (10^{-7} M)	IC_{50} (-log M)	B_{MAX} (fmol/mg protein)
Ketanserin	(5-HT_{2A} antagonist)	1.9	7.91 ± 0.09	140
Mianserin	(5-HT antagonist)	2.0	7.89 ± 0.06	120
Ritanserin	(5-HT_2/5-HT_{1C} antagonist)	2.2	7.65 ± 0.54	102
Serotonin	(neurotransmitter)	2.8	7.3 ± 0.49	80.6
Spiperone	(D_2/5-HT_2 antagonist)	2.8	7.2 ± 0.21	79
(±)-2-5-Dimethoxy-4-iodoamphetamine hyrobromide (DOI)	(5-HT_2/5-HT_{1C} agonist)		6.8 ± 0.21	62.8
Methiothepin mesylate	(5-HT_1 antagonist)	3.54	6.9 ± 0.67	58
Metergoline	(5-HT_1 antagonist)		6.7 ± 0.58	ɔ1
2-Methyl-5-HT-maleate	(5-HT_3 agonist)		5.7 ± 0.76	38
α-Methyl-5-HT-maleate	(5-HT_2 agonist)		5.8 ± 0.12	
5-HIAA	(5-HT metabolite)	20	5.1 ± 0.28	
Reserpine	(amine depletor)	2600	5.3 ± 0.32	

Note: [^{3}H]Ketanserin was incubated with juvenile trout hypothalamus membrane preparation in the absence (B_o) and presence (NSB) of radiostable ketanserin to determine specific binding (B_{sp}). [^{3}H]Ketanserin was displaced differentially by various competitors, depending on their binding affinity and capacity for the 5-HT_2-like binding site in the tissue sample. Experiments were conducted in triplicate and data were expressed as the mean $\pm$ SEM. Scatchard plots (Scatchard 1949) were used to determine K_D (K_i) values for each competitor (except 5-HIAA and reserpine) and the Half-maximal inhibitory concentration (IC_{50}) values for each competitor were estimated from logit-log plots by plotting logit (logit = ln[P/(100 –P)], P is percent bound) of total [^{3}H]ketanserin binding to trout hypothalamic membrane preparation versus -log [competitor, M]. The IC_{50} is 50% binding, and the logit of 50 % [ln (1)] is 0. Thus, the IC_{50} was determined by linear correlation (Bylund and Yamamura 1990) (not shown). K_i values were comparable to those calculated according to Cheng & Prusoff (1973), $K_i = IC_{50} / [1 + C / K_D]$, where C is concentration of radioligand and K_D is dissociation rate constant obtained from saturation experiment. Comparison of binding affinity and specificity of the various structurally related competitors reveals a rank order of potency of ketanserin = mianserin > ritanserin > 5-HT = spiperone >> methiothepin mesylate > metergoline = DOI > 2-methyl-5-HT > α-methyl-5-HT >>>>5-HIAA = reserpine.

CHAPTER 3.

*Levels of specifically bound [^{3}H]ketanserin compared to levels of 5-HT in the brain regions of juvenile and sexually recrudescing female rainbow trout (*Oncorhynchus mykiss*).*

Levels of specifically bound [^{3}H]ketanserin compared to levels of 5-HT in the brain regions of juvenile and sexually recrudescing female rainbow trout *Oncorhynchus mykiss*.

Abstract

This study compared the distribution of specifically bound [^{3}H]ketanserin (B_{sp}) with serotonin (5-HT) in brain regions of juvenile and sexually recrudescing female trout. Amounts of B_{sp} varied widely among brain regions and consistently differed between juvenile and sexually recrudescing females. Levels of B_{sp} were significantly greater in the hypothalamus than the olfactory lobe, which were at least three-fold greater than all other tissues examined (Kruskal Wallis test, $p<0.05$). B_{sp} densities in the hypothalamus, pre-optic area, and optic lobe were significantly greater in juveniles compared with corresponding tissues from sexually recrudescing females (Mann Whitney-U test, $p<0.05$); in contrast, B_{sp} in olfactory lobe and spinal cord did not differ significantly between the two classes of fish. 5-HT concentration was determined by HPLC-EC analysis. Biogenic amine standards eluted in a stereotypic pattern, with peaks consistently separable in time. 5-HT concentration was significantly greater in hypothalamus than in olfactory lobe and undetectable in the pituitary (Kruskal Wallis test, $p<0.05$). Trends in distribution of B_{sp} and 5-HT were comparable in the hypothalamus and pre-optic area in juvenile and sexually recrudescing females. In general, density of specific [^{3}H]ketanserin binding sites was directly related to 5-HT content of brain regions in juvenile and sexually recrudescing females. 5-HT concentrations (pmoles/g tissue) were approximately 900-fold greater than B_{sp} (fmoles/g tissue) in all brain regions, and approximately 300-fold greater than B_{sp} in the olfactory lobe. These results suggest important regulatory role(s) for 5-HT in the trout pre-optic-

hypothalamo-hypophysial axis, which may differ from 5-HT role(s) in trout olfactory lobe.

Key Words: High Performance Liquid Chromatography-Electrochemical Detection, [^{3}H]ketanserin, and sexually recrudescing female trout.

Introduction

Expression of biological activity of neuroactive substances depends largely on co-localization of the primary messenger, such as serotonin (5-HT), with its specific receptor. Examination of regional distribution and density of messengers, such as 5-HT, and their receptors can reveal important aspects of their inter-dependence, and may predict localization of messenger biological activity. Previously we have described the existence and binding characteristics of a specific [^{3}H]ketanserin binding site in the hypothalamus of rainbow trout *Oncorhynchus mykiss* (Agrawal and Omeljaniuk 1999, submitted). The binding characteristics of this site are reminiscent of the mammalian 5-HT_2 receptor (Sanders-Bush and Mayer 1996, Leysen and Pauwels 1990, Leysen et al. 1981). Now we use this radioreceptor assay to survey various brain regions and the pituitary in juvenile and sexually recrudescing female rainbow trout to determine the relative abundance of this binding site. As well, we use the technique of high-performance liquid chromatography with electrochemical detection (HPLC-EC) to determine the content of 5-HT, other biogenic amines, and their metabolites in these same brain regions.

Serotonergic neuron and fiber distribution has been extensively studied in brain regions of various species of bony fish (teleosts). A large number of 5-HT cell bodies are found in the midbrain, brain stem and diencephalon of goldfish (*Carassius auratus*; Kah and Chambolle 1983), African catfish (*Clarias gariepinus*; Corio et al. 1991) and rainbow trout (*Salmo gairdneri*; Frankenhuis-van den Heuvel and Nieuwenhuys 1984). Particularly high densities of 5-HT have been found in the nucleus raphe medialis in various species of teleosts including the African catfish (*C. gariepinus*; Corio et al. 1991), three spined stickleback (*Gasterosteus aculeatus* L; Ekstrom and Van Veen 1984), sunfish (*Lepomis*

gibbosus; Parent et al. 1978), and sockeye salmon (*Oncorhynchus nerka* Walbaum; Ekstrom and Ebbesson 1989). The raphe nucleus in the three spined stickleback (*G. aculeatus* L) projects large numbers of serotonergic axons to multiple regions of the brain including the ventral thalamus, hypothalamus, and pituitary with a few scattered varicosities in the cerebellum (Ekstrom and Van Veen 1984). Small populations of serotonergic neurons are found in other goldfish (*C. auratus*) brain regions including, olfactory lobe, pre-optic area and optic lobe (Kah and Chambolle 1983) and fibers from the raphe nucleus of the midbrain, extend to the pars distalis of the pituitary gland, medulla oblongata, spinal cord, the olfactory lobes (Kah and Chambolle 1983). The abundance of 5-HT in these regions implicates the presence of a teleost 5-HT receptor therein.

Serotonin is an important neuroendocrine factor in teleost brain regions. For example, 5-HT inhibits secretion of growth hormone (GH) (in a dose-related manner) and causes a dose-related release of gonadotropin (GtH) (Somoza and Peter 1991) from *in vitro* perifused pituitary fragments of goldfish (*C. auratus* L). By comparison, 5-HT stimulates release of maturational GtH from the pituitary of female Atlantic croaker (*Micropogonias undulatus*) *in vivo* and *in vitro* (Khan and Thomas 1992); similarly, 5-HT increases plasma GtH levels in both female and male goldfish (*C. auratus* L) *in vivo* (Somoza et al. 1988). In the pituitary of mollies (*Poecilia latipinna*), 5-HT mildly stimulates secretion of GtH in both males and females at different stages of reproduction (Groves and Batten 1985), indicating serotonergic regulation of reproduction. In rainbow trout, Saligaut et al. (1992) demonstrated physiological fluctuations in 5-HT levels during ovarian recrudescence and ovulation as well as simultaneous brain 5-HT and dopamine (DA) turnover at various reproductive cycles of the female rainbow trout, implying

regulatory roles for 5-HT and DA in female rainbow trout reproduction. Comparable annual and daily variations in serotonin levels were demonstrated in the hypothalamus of the Indian catfish (*Heteropneutes fossilis*) *in vivo*, during gonadal recrudescence or gonadal dormancy (Senthilkumar and Joy 1993). Collectively, these data strongly implicate the presence of a 5-HT-receptor site in teleost brain and pituitary regions and suggest an extensive age-related, serotonergic regulation of the teleost hypothalamic-pituitary axis. These findings also suggest possible significant variation in 5-HT and 5-HT receptor dynamics in this region.

Our present research investigates distribution of 5-HT and a specific [^{3}H]ketanserin binding site in the brain:pituitary axis of rainbow trout and examines the impact of sexual maturity on these parameters.

Materials and methods

Experimental animals

Fingerling rainbow trout (*Oncorhynchus mykiss*; Rainbow Springs Trout Hatchery, Thamesford, Ont.) were raised to juveniles in the Lakehead University Aquatic Animal Research Facility in flow-through aquaria with dechlorinated water at simulated ambient temperature (annual range, 5 to 16^0C) and photoperiod (annual range, 8 to 14h photophase). Fish were fed commercial trout pellets daily (1 to 3% body weight; Zeigler trout feed, Thunder Bay Co-Op), and were assorted into two groups: juveniles and sexually recrudescing females. Juvenile fish (38 $\pm$ 8 cm long) were approximately 24 months old (and did not possess obvious gonads) whereas sexually recrudescing female fish (65 $\pm$ 8 cm long) were approximately 36 months old (were gravid, and possessed obvious ovaries). Any of the few fish with the obvious presence of testes were excluded from this study to keep the brain pool relatively constant in every trial. All fish were maintained and handled in accordance with guidelines established by the Canadian Council on Animal Care as well as the Ontario Animals for Research Act. In all cases, fish were anesthetized with tricaine methanesulphonate (MS-222, 0.5g/liter; Syndel Laboratories, Vancouver, B.C) prior to any handling, then killed by spinal transection posterior to the medulla oblongata.

Tissue preparation for radioligand binding assay

Individual tissue preparations were created for every independent experiment in this study. Whole brains were removed and placed in ice-cold assay buffer (AB; 50 mM Tris-HCl, pH = 7.4). For a given assay, olfactory lobe, hypothalamus, pituitary, pre-optic area (just superior and dorsal to the hypothalamus, and ventral and inferior to the optic lobes; it was

dissected from the ventral side of the brain, after the hypothalamus had been just removed), optic lobe, and spinal cord, identified on the basis of Billiard and Peter (1982), were surgically isolated by microdissection. For each experiment replicate tissue samples were pooled into individual Corning 15-ml polystyrene centrifuge tubes, suspended in liquid N_2. These pools of different tissue types were stored in liquid N_2 until the next day for inclusion in the radioligand binding protocol.

[^{3}H]Ketanserin binding assay

The [^{3}H]ketanserin binding assay was previously developed in our lab (Agrawal and Omeljaniuk 1999, submitted) based on a modification of Leysen et al. (1982). Frozen tissue was transferred to an ice-cold glass mortar and combined with (100 µl/original tissue sample) ice-cold, homogenization buffer (HB: 50 mM Tris-HCl, pH 7.4; 0.32 M sucrose; Gallaher and Wang 1990; Leysen et al. 1982); all subsequent procedures were carried out at 0 to 4^{0}C. Tissue was homogenized with ten strokes of a motor-driven Potter-Elvehjem homogenizer (0.125 mm clearance) and homogenate was transferred to 10-ml polypropylene centrifuge tubes. Homogenate was centrifuged at 1000g (20min), the supernatant aspirated and transferred to Beckman Ultra-Clear centrifuge tubes (13 x 32mm) and centrifuged at 100,000g (30min). The resulting supernatant was decanted to waste and pellet was homogenized in AB (100 µl/ original tissue sample) and centrifuged at 100,000g (30min). To prepare the membrane suspension, the resulting supernatant was decanted to waste and pellet suspended in AB (100 µl/ original tissue sample).

Typically, a 100µl aliquot of membrane suspension was incubated with 100µl [^{3}H]ketanserin (NEN-Dupont, Boston, MA; 61 Ci/mmol) and 100µl AB (to estimate total binding (B_o)) or 100µl competitor (10µM unlabelled ketanserin), to estimate non-specific

binding (NSB), resulting in a final volume of 300µl. Specifically bound [^{3}H]ketanserin (B_{sp}) was calculated as the difference between B_o and NSB. Binding reactions were terminated by filtration through Whatman GF/B filters (CanLab, Vancouver, BC.), presoaked overnight in AB containing 0.3% polyethyleneimine to reduce nonspecific binding (Schwartzentruber and Omeljaniuk 1994), followed by 3 rinses of 3 ml ice-cold assay buffer. Filters were placed in 6-ml scintillation vials (Beckman, Mississauga, ON) and incubated overnight in 4 ml ReadySafe scintillation cocktail (Beckman, Mississauga, ON); radioactivity was determined by liquid scintillation spectroscopy at 50% counting efficiency. [^{3}H]ketanserin bound optimally to trout hypothalamic membrane preparation within the range of 0.5 ± 0.02 to 1.5 ± 0.3 mg protein/ml (Agrawal and Omeljaniuk 1999 submitted); all subsequent tissue preparations were diluted to within this range.

Data Analysis for radioligand binding assay

Data were derived from 4 independent experiments (4 replicates/experiment) and expressed as mean $\pm$ SEM. B_{spMEAN} was calculated as the difference between B_{oMEAN} and NSB_{MEAN}; B_{spSEM}, in comparison, was calculated as $(B_{oSEM}^2 + NSB_{SEM}^2)^{1/2}$ (Hulme and Birdsall 1992). Specifically bound [^{3}H]ketanserin has been presented as cpm/mg protein for each tissue type for comparison with values in Agrawal and Omeljaniuk (1999 submitted). B_{sp} is also expressed as pmoles/g tissue for comparison with biogenic amine content in brain tissues (fmoles/g tissue). B_{sp} of tissues was statistically compared among tissue types within a class of animals on the basis of Kruskal Wallis test ($p<0.05$), and between juveniles and sexually recrudescing females in corresponding tissue types on the basis of Mann Whitney-U test ($p<0.05$).

Tissue preparation for high-performance liquid chromatography analysis

Whole brains were removed and placed on an ice-cold petri dish. The hypothalamus, pre-optic lobe, olfactory lobe, and pituitary gland were isolated by microdissection, then individually transferred to pre-weighed 1.5-ml polyethylene centrifuge tubes suspended in liquid N_2. Tissue was stored in liquid N_2 for not more than two hours, before being processed for HPLC-EC analysis.

High-performance liquid chromatography with electrochemical detection of 5-HT and other biogenic amines

The protocol for high-performance liquid chromatography (HPLC) analysis was derived from Sloley et al. (1991) with few modifications. All steps were conducted at 0 to 4°C. Frozen tissue samples were individually sonicated in their centrifuge tubes in the presence of 500 µl (200µl for pituitary) perchloric acid ($HClO_4$; 0.2M) with a Branson Ultrasonic Tissue Disruptor (70 Watts, 20khz, for 15 seconds). Sonicates were centrifuged at 12,800g (10 min) and supernatant aspirated and centrifuged at 12,800g (10 min) to ensure complete removal of tissue particles and precipitated proteins. Isoproterenol (300pg in 10 µl) was added to all sample extracts as an internal standard (Sloley et al. 1991). Aliquots (10µl) of sample extracts were applied to the HPLC column using a Shimadzu SIL-10A automatic sample injector.

Standard solutions of serotonin (5-HT), dopamine (DA), epinephrine (E), homovanillic acid (HVA; DA metabolite), 3, 4-dihydroxyphenylacetic acid (DOPAC; DA metabolite), norepinephrine (NE), and 5-hydroxyindole-acetic acid (5-HIAA; 5-HT metabolite) (all purchased from Research Biochemicals Inc., Natick, MA), were freshly prepared in 0.2M $HClO_4$ in concentrations ranging from 30pg/10µl to 900pg/10µl for

each experiment. The HPLC mobile phase, included 75mM NaH_2PO_4, 1mM sodium octyl sulphate and 0.05 mM EDTA, was prepared in double-distilled, deionized water with a resistance approximately 18.3 MΩ-cm (Barnstead NANOpure Ultrapure water system) and filtered prior to addition of acetonitrile (final concentration, 13% v/v) to make up the final volume. pH was adjusted to 2.75 with concentrated phosphoric acid, and the mobile phase was degassed at 1 ATM for at least 24 hrs before use in the HPLC system.

All chromatographic separations were performed through a Beckman Ultrasphere ODS column (10 x 0.46 cm, 3-μm particles). The HPLC system consisted of an LC-10AS HPLC pump coupled with an SIL-10A model automatic sample injector, and regulated by a Shimadzu SCL-10A system controller. An ESA (Bedford, MA) model 5100A Coulochem detector was used for electrochemical detection of eluting species. The filter time constant was set to 2s and sensitivity was usually set at 35 x 10 (nA). The ESA 5100A system included a model 5011 dual analytical cell and a model 5020 guard cell. Guard cell voltage was set at + 0.43 V, and detector 2 at + 0.43V. Chromatographic data were acquired and stored by Shimadzu EZChrom software (1994). During each separation, the mobile phase was pumped at a flow rate of 1ml/min (Sloley et al. 1991) and pressure of 165 ± 2 atmospheres.

Data Analysis for HPLC

Chromatography peaks for 5-HT, DA, HVA, DOPAC, 5-HIAA, NE, E and isoproterenol were identified on the basis of their retention times derived from repeated analyses (n=8, with 2 repetitions per experiment). All the detected species were quantitated based on peak height. % CV in the elution pattern of the biogenic amines were estimated on the basis of internal standard (isoproterenol) elution levels. This method of estimation is

consistent with previous studies (Sloley et al. 1992, Dulka et al. 1992). Although, low C V values (approximately 6.6 %), suggested low variability in elution levels of the internal standard between samples and standards, values for chemical species were calculated on the basis of standard curves for each experiment. This method suggested accurate detection in our study, with low margin of error. Tissue levels of biogenic amines were determined in each HPLC analysis session on the basis of standard curves for each species; results were expressed in terms of ng/g wet-weight of tissue. 5-HT concentration in tissues was statistically compared among tissue types within a class of animals on the basis of Kruskal Wallis test ($p<0.05$); and between juveniles and sexually recrudescing females in corresponding tissue types on the basis of Mann Whitney-U test ($p<0.05$). All statistical estimations were based on the SPSS/PC$^+$ computer software package.

Protein content

As individual fish pituitaries were difficult to weigh accurately, estimates of specifically bound [^{3}H]ketanserin and 5-HT content were related to protein content, determined by the Bradford method (Bradford 1976) using Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, CA) and bovine serum albumin (Sigma Chemicals, St. Louis, MO) as a protein standard.

Results

Amounts of specifically bound [^{3}H]ketanserin (B_{sp}) varied among trout brain regions (Table 1). In general, levels of B_{sp} in the hypothalamus were significantly larger than in the olfactory lobe, which, in turn, were at least three-fold greater than in all other tissues examined (Kruskal Wallis test, $p<0.05$) (Table 1). In juveniles, highest B_{sp} levels were detected in the hypothalamus (1620 $\pm$ 109 cpm/mg protein), which were larger than B_{sp} levels in the olfactory lobe (987 $\pm$ 67 cpm/mg protein), which in turn were larger than levels in the optic lobe, pre-optic area and spinal cord (Table 1). Similarly, in sexually recrudescing females, highest B_{sp} levels were detected in the hypothalamus (1100 $\pm$ 127 cpm/mg protein), which were larger than B_{sp} levels in the olfactory lobe (423 $\pm$ 34 cpm/mg protein), which in turn were larger than levels in the optic lobe, pre-optic area and spinal cord (Table 1). Binding site densities in the hypothalamus, pre-optic area, and optic lobes, and hypothalamus were significantly greater in juveniles compared with corresponding tissues from sexually recrudescing females (Mann-Whitney U test, $p<0.05$). In contrast, binding site densities in the spinal cord did not differ significantly between juveniles and sexually recrudescing females (Mann-Whitney U test, $p<0.05$) (Table 1). Amounts of protein detected in the pituitaries of both juveniles and sexually recrudescing females were almost vanishingly small hence levels of B_{sp} detected in each were relatively low at 1.92 $\pm$ 0.63 and 3.26 $\pm$ 1.4 cpm/μg protein respectively (data not shown).

HPLC-EC analysis of biogenic amine standards resulted in a stereotypic elution pattern; peaks were consistently separable in time (Fig. 1), and had highly conserved retention times (Table 2). The order of elution of chemical species from our system was norepinephrine, epinephrine, DOPAC, dopamine, 5-HIAA, HVA, and 5-HT (Table 2).

Varied amounts of standards were examined for electroactivity; based on these values, standard curves were generated and quantified by linear regression analysis (Fig 2). Standard curves for chemical species were grouped and represented according to their closest biosynthetic and metabolic origins (Fig. 2 panels a, b, and c). Linear regression analysis for data from each standard curve consistently resulted in r^2 values ≥ 0.996. Chemical species in tissue samples were identified based on their retention time; a typical chromatogram is depicted in Fig 1. As in the standards, peak overlap was not observed between any chemical species. The amount of each chemical specie present in fish samples was estimated based on relevant standard curves.

Concentrations of chemical species varied widely among brain regions and the pituitary of juvenile and sexually recrudescing female trout (Table 3). In juveniles, 5-HT concentration was three to four fold greater in the hypothalamus (228 $\pm$ 12 ng/g tissue) than in both the pre-optic area (56 $\pm$ 7 ng/g tissue) and olfactory lobe (78 $\pm$ 13 ng/g tissue). Similarly, in sexually recrudescing females, 5-HT concentration was greater in the hypothalamus (93 $\pm$ 7 ng/g) than both pre-optic area (21 $\pm$ 2 ng/g) and olfactory lobe (73 $\pm$ 20 ng/g). 5-HT concentration was significantly greater in the hypothalamus of juveniles compared with sexually recrudescing females (Mann Whitney-U test, $p<0.05$). Although 5-HIAA levels were usually greater than 5-HT in both juvenile and sexually recrudescing female tissue types (Table 3), no obvious correlation could be derived for the distribution of the neurotransmitter and its metabolite. Limited tissue availability precluded larger scale pituitary analyses.

DA levels in the hypothalamus were significantly greater than in both olfactory lobe and pre-optic area in both classes of trout (Kruskal-Wallis test, $p<0.05$) (Table 3). In

juveniles, DA levels were approximately two-fold greater in the hypothalamus, olfactory lobes and pre-optic areas compared with corresponding tissues from sexually recrudescing females. DA levels in juvenile pituitary were quite low, but comparable with levels in sexually recrudescing females. In juvenile and sexually recrudescing females, either DOPAC, HVA, or both, were detected (much lower levels) in brain regions corresponding to DA, however, no apparent correlations could be made about the distribution of the neurotransmitter and its metabolites. Comparable levels of NE were detected in the hypothalamus and olfactory lobes of trout brain. These levels were significantly greater (approximately two-fold) than those detected in the pre-optic area in both classes of trout (Kruskal-Wallis test, $p<0.05$) (Table 3). NE levels were consistently greater in brain regions of juveniles compared with corresponding regions in sexually recrudescing females. In all cases, levels of epinephrine were below detection limits (data not shown).

In general, tissue 5-HT levels (pmoles/g tissue) were greater in juveniles, than in corresponding brain regions of sexually recrudescing females, with the exception of the olfactory lobe, where 5-HT levels were comparable between juveniles and sexually recrudescing females (Fig. 3 panel A). B_{sp} levels (pmoles/g tissue) were significantly greater in juvenile hypothalamus, pre-optic area and olfactory lobe than in corresponding regions of sexually recrudescing female trout (Mann-Whitney U test, $p<0.05$) (Fig. 3 panel B). The ratio of 5-HT content to specifically bound [^{3}H]ketanserin in all trout brain regions was collectively 947 ($\pm$ 240):1 with the exception of juvenile olfactory lobe where 5-HT content was approximately 300-fold greater than specifically bound [^{3}H]ketanserin (Fig. 3 panel A and B).

Discussion

Colocalization of neurotransmitters with their respective receptors is a necessary requirement for expression of neurotransmitter biological activity. The teleost (bony fish), brain:pituitary axis constitutes a powerful model for investigation of neurotransmitter:receptor interaction and their mutual regulation. The teleost pituitary gland is directly innervated by neurosecretory axons originating from the hypothalamus. This feature, and the absence of a functional hypothalamo-hypophysial portal system, makes teleosts a unique experimental model (Fryer and Maler 1981, Peter et al. 1990, Anglade et al. 1993). Direct innervation of individual cells allows for a precise hypothalamic regulation of pituitary hormone secretion and implies the presence of various neuroendocrine receptors in this axis (Peter et al. 1990). The power and utility of this model is illustrated in part by examination of the distribution of serotonin and its receptors in the teleost brain:pituitary axis, and the influence of serotonin on release of pituitary hormones associated with reproduction and stress-response physiology. For example several prominent regions of the teleost brain:pituitary axis participate in 5-HT bioactivity. Frequently cited regions include, olfactory lobe of three spined stickleback (*Gasterosteus aculetus* L. Ekstrom and Van Veen 1984), hypothalamus of goldfish (*C. auratus* L, Kah and Chambolle 1983), pre-optic area of catfish (*Clarias gariepinus* Corio et al 1991), and rainbow trout (*Salmo gairdneri*, Frankenhuis-van den Heuvel and Nieuwenhuys 1984), as well as the pituitary gland of mollies (*Poecilia latipinna*, Groves and Batten 1985), and goldfish (Kah and Chambolle 1983). In this study we present evidence on the differential distribution of 5-HT_2-like receptors in elements of the teleost brain:pituitary axis that have been implicated in regulating release of reproductive and stress-response hormones.

Moreover, we present data on the influence of reproductive status of receptor density and relationship of local 5-HT content and receptor density.

The first direct evidence for existence of a teleost (CNS) 5-HT_2 receptor was based on the ligand recognition criteria and specific binding of [^{3}H]ketanserin, a selective mammalian 5-HT_2 antagonist, to rainbow trout hypothalamic membrane preparation (Agrawal and Omeljaniuk 1999 submitted). Various regions of trout brain are constituents of a neural pathway, which figures prominently in brain regulation of "brain:pituitary:gonadal" function (Anglade et al. 1993, Corio et al. 1991); this makes these regions ideal candidates in this study. For example, prominent hypophysiotrophic areas include the pre-optic area, hypothalamus and pituitary in the brain regions of goldfish (Anglade et al. 1993), and African catfish (Corio et al. 1991). In this study, amounts of specifically bound [^{3}H]ketanserin varied among trout brain regions (Table 1). B_{sp} in trout hypothalamus was significantly larger than in olfactory lobe (Fig. 3, Panel B), which, in turn were at least three-fold greater than in all other regions examined. Rank order of binding density in trout brain regions was hypothalamus > olfactory lobe >>> pre-optic area > spinal cord > optic lobe >>> pituitary. B_{sp} levels in trout hypothalamus were comparable with previous results (Agrawal and Omeljaniuk 1999, submitted), implying the reliability of this protocol between independent studies. In rat brain, by comparison, the rank order of specifically bound [^{3}H]ketanserin density was prefrontal cortex (23.7 $\pm$ 0.5 pmoles/g tissue) >> temporal cortex (10.7 $\pm$ 2 pmoles/g tissue) >>>>>> hypothalamus (0.7 $\pm$ 0.4 pmoles/g tissue) >>>> pituitary (undetectable) (Leysen et al. 1982). Similar trends were found in guinea pig (Leysen et al. 1982 and Leysen et al. 1983) and human (Schotte et al. 1983) brain regions.

In our trout, pituitary levels of B_{sp} in both juvenile and sexually recrudescing females were 1.92 ± 0.63 and 3.26 ± 1.4 cpm/μg protein respectively (data not shown). These levels are fairly large when expressed as cpm/mg protein. However, this detection is limited by the extremely small amount of tissue associated with individual trout pituitaries. Also, the signal:noise ratio is low even for pooled samples of trout pituitaries. By comparison, B_{sp} levels are undetectable in rat pituitary and comparatively very low in guinea pig (0.5 ± 0.2 pmoles/g tissue) (Leysen et al. 1982). Collectively these results suggest that the relatively larger density of binding sites in the trout pituitary is indicative of a relatively more prominent role for 5-HT in the pituitary of teleosts than mammals. This observation is consistent with our demonstration of large numbers of binding sites in trout brain regions implicated in growth and reproduction. For example, 5-HT stimulates gonadotrophs in the hypothalamus and pre-optic area in, Atlantic croaker (*M. undulatus*, Khan and Thomas 1992), in goldfish (*C. auratus*, Somoza and Peter 1991; Somoza et al 1988; Yu et al. 1991) and in male and female mollies (*P. latipinna*, Groves and Batten 1985). 5-HT also inhibits growth hormone (GH) release in goldfish pituitary (Somoza and Peter 1991).

Levels of B_{sp} varied significantly in the olfactory lobe, optic lobe, pre-optic area and hypothalamus between juvenile and sexually recrudescing females (Table 1). Although there is no direct previous information which compares levels of specifically bound [^{3}H]ketanserin between juvenile and sexually recrudescing females in teleost models, indirect evidence suggest that 5-HT regulates factors governing teleost growth and reproductive status. In teleost hypothalamus, gonadotropic releasing hormone (GnRH) releasing neurons project fibers into gonadotrophs (Peter et al. 1990) located in the

pituitary, which, in turn, release gonadotropic hormone (GtH) into circulation. Previous investigations suggest serotonin has dose dependent stimulatory effects on immunoreactive-GnRH release from goldfish pre-optic area:hypothalamus brain slices and GtH from pituitary fragments, suggesting, serotonergic inputs at both the pre-optic and pituitary levels of the GnRH:GtH system (Yu and Peter 1990). 5-HT stimulates GtH release in goldfish both *in vivo* (Somoza et al. 1988) and *in vitro* from perfused fragments of the pituitary (Somoza and Peter 1991); pretreatment of fish with ketanserin blocked the stimulatory effects of 5-HT on serum GtH levels in both female and male goldfish (Somoza et al.1988) suggesting involvement of 5-HT_2-like binding sites in the release of GtH. In 1-year old Atlantic croaker (*M.undulatus*, Khan and Thomas 1992), intraperitoneal administration of the combination of leutinizing hormone releasing hormone (LHRHa) and 5-HT elicited an increase in GtH levels, which were significantly greater than that induced by LHRHa or 5-HT alone. In comparison, 5-HT alone or in combination with LHRHa, stimulated GtH release both *in vitro* and *in vivo* using sexually mature croakers (Khan and Thomas 1992). Our findings collectively support the concept of 5-HT as a major neuroendocrine regulator of the brain:pituitary gonadal axis and suggest that this 5-HT regulation may vary as a function of sexual maturity.

Our biogenic amine analysis was reliable and yielded results comparable with previous studies (Hernandez-Rauda et al. 1996, Sloley et al 1992, Saligaut et al 1990, 1992, and Dulka et al. 1992). Retention times for all the detected biogenic amines in our study (Table 2) were comparable to previously observed values (Hernandez-Rauda et al. 1996) derived from HPLC-EC analysis using comparable mobile phase composition. In studies where similar mobile phase was used at different pH values (Hall et al. 1989, Cox

Gariepy et al. 1994) retention times were found to vary, however, the elution order of chemical species were similar. Chemical species eluted in a consistent order, with the fastest elution of norepinephrine (2.14 $\pm$ 0.021 min.) and slowest of 5-HT (7.27 $\pm$ 0.112 min.) (Fig 1 and Table 2). Standard curves used for biogenic amines were highly predictable and reproducible with very small interassay variability (< 5%), emphasizing the reliability of this technique.

In our trout, (both juvenile and sexually recrudescing female), 5-HT concentration was found to be greater in the hypothalamus (228 $\pm$ 12 ng/g tissue; 93 $\pm$ 7.3 ng/g tissue), than in both the pre-optic area (56 $\pm$ 7.3 ng/g tissue; 21 $\pm$ 3 ng/g tissue) and olfactory lobe (78 $\pm$ 13 ng/g tissue; 73 $\pm$ 20 ng/g tissue) (Table 3). By comparison, Saligaut et al. (1992) detected similar trends in 5-HT levels with higher levels of 5-HT (approximately 500 ng/g tissue) in the hypothalamus of sexually recrudescing female rainbow trout, compared to the pre-optic area (approximately 150 ng/g tissue). Actual levels of 5-HT detected in our study were much lower compared to corresponding brain tissues in the Saligaut et al. (1992) study. Overall, levels of 5-HT and other biogenic amines were higher in juveniles than sexually recrudescing females (Table 3). This trend is consistent with levels of B_{sp} in juvenile versus sexually recrudescing-female trout (Fig. 3). Saligaut et al (1992) observed an increase in 5-HT content in trout hypothalamus during the pre-ovulatory period, suggesting increased 5-HT synthesis and release in this region of the brain. This observation is consistent with the age-related changes in the distribution of 5-HT in the forebrain and pituitary observed in platyfish during reproductive senescence (Margolis-Nunno et al. 1986). Collectively these results imply a role for 5-HT in the regulation of teleost sexual maturation and ovulation. In rats by contrast, highest 5-HT levels were

detected in the hippocampus (8.1 ± 3.58 pg/30ul sample), (Cox Gariepy et al. 1994, Acworth et al. 1994), striatum (15 fold less than in hippocampus) (Wong et al. 1995), with no detectable levels in hypothalamus or pre-optic area. Collectively this information suggests that 5-HT may play a more prominent role in the teleost preoptic area:hypothalamus axis than it does in mammals. In contrast to our findings in the brain, 5-HT levels were undetectable in trout pituitary (Table 3); this finding is consistent with those of Saligaut (1992) who failed to reliably measure 5-HT in female trout pituitary at any stage of its reproductive cycle.

DA levels were also high in trout hypothalamus compared with other brain regions, both in juveniles and sexually recrudescing females (Table 3). Our finding is comparable with the high DA levels observed in goldfish hypothalamus (656 ± 55 ng/g) (Dulka et al. 1992) compared with other brain regions. In mammals, high DA levels have been observed in rat hippocampus (Cox Gariepy et al. 1994) with basal levels of 6.3 ± 0.5 "pg/collection" (Acworth et al. 1994), rat striatum (Wong et al. 1995) with basal levels of 51.8 ± 6.8 pg/collection, and rat cortex (Alburges et al. 1993). Collectively our findings support the concept that DA and 5-HT may act as coordinate neuroregulators in these neuroendocrine pathways.

Neurotransmitter:receptor ratios for 5-HT and Bsp were highly predictable throughout the trout brain in both juvenile and sexually recrudescing females, with the exception of the juvenile olfactory lobe (Inset to discussion, Fig 4). To the best of our knowledge, this investigation is the first ever to compare levels of 5-HT to its specific binding site in trout brain. B_{sp} values (fmoles/g tissue) were plotted as a function of 5-HT content (pmoles/g tissue) using data points including all tissue types from both juvenile

and sexually recrudescing female fish, with the exception of juvenile olfactory lobe and analyzed by linear regression analysis. The very high correlation of Bsp with 5-HT content (r^2= 0.994) suggests a direct influence of 5-HT on the density of specific [^{3}H]ketanserin binding sites in these brain regions, within the ages and sexual status of the trout examined. Differing 5-HT:B_{sp} ratio in the olfactory lobe suggest that 5-HT regulation of the olfactory lobe might differs from that in the pre-optic-hypothalamo-hypophysial axis.

To conclude, the primary findings of this research include, high B_{sp} levels and 5-HT content in trout hypothalamus compared to other brain regions, suggesting important regulatory role(s) of 5-HT in the hypothalamus of rainbow trout. Both, B_{sp} and 5-HT levels were higher in juvenile brain regions compared to corresponding regions in sexually recrudescing females suggesting age-related changes in 5-HT regulation of growth and sexual maturity in rainbow trout brain. 5-HT content (pmoles/g tissue) was directly related to B_{sp} levels (fmoles/g tissue) suggesting a region specific relation between neurotransmitter and 5-HT_2 levels in rainbow trout brain regions. Collectively these results suggest that levels of specific [^{3}H]ketanserin binding and 5-HT may be mutually predictive in these trout brain regions.

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Fig. 1. Electroactivity of eluate (Volts) for biogenic amine and metabolite standards plotted as a function of time (min). Panel A is a typical chromatogram for biogenic amine and metabolite standards at 900pg; elution pattern is as follows: NE, E, DOPAC, DA, 5-HIAA, HVA, and 5-HT. Panel B shows a chromatogram for juvenile hypothalamus with chemical species identified according to elution pattern. Species identified are as follows: NE, DA, 5-HIAAA, HVA and 5-HT. Levels of other chemical species were too low to be detected by this method.

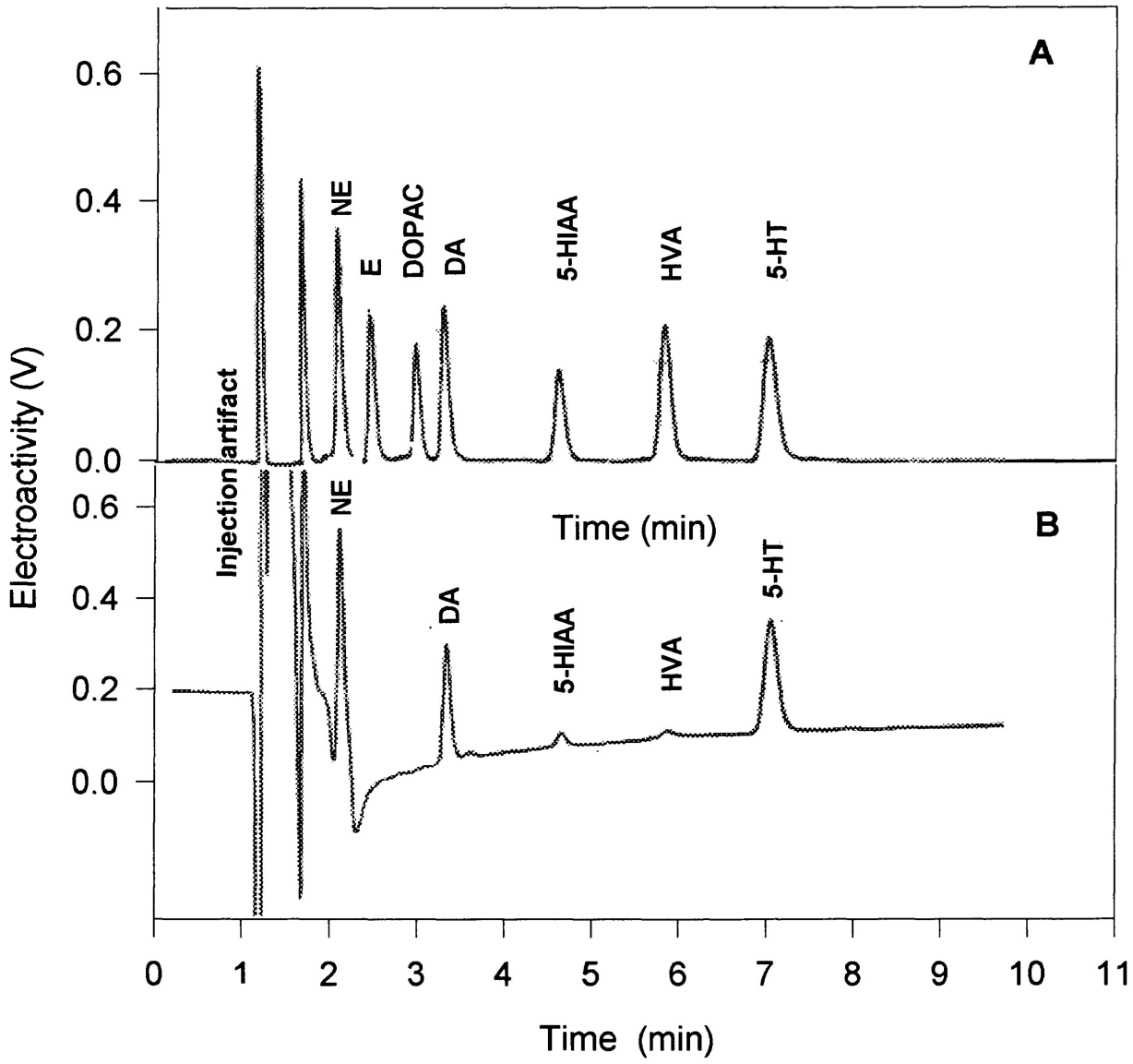
A
B
Electroactivity (V)
0.6
0.4
0.2
0.0
Injection artifact
NE
E
DOPAC
DA
5-HIAA
HVA
5-HT
Time (min)
0
1
2
3
4
5
6
7
8
9
10
11
Time (min)

Fig. 2. Standard curves for biogenic amine and selected metabolite standards where electroactivity (V) is plotted as a function of amount of standard (pg). Species are grouped according to biological activity and values are means (± SEM). In all cases linear regression of the data resulted in r^2 values > 0.996. Panel a, serotonin **(5-HT)**, peak height (V) = $(2.55 \times 10^{-4}) \times$ (5-HT (pg)) + 5.4×10^{-3} ($r^2 = 0.99$); and its metabolite 5-hydroxyindoleacetic acid **(5-HIAA)**, peak height (V) = $1.7 \times 10^{-4} \times$ (5-HIAA(pg)) + 0.01×10^{-3} ($r^2 = 0.98$). Panel b, dopamine **(DA),** peak height (V) = $3.049 \times 10^{-4} \times$ (DA(pg)) + 1.74×10^{-3} ($r^2 = 0.99$); and its metabolites 3, 4-dihydroxyphenylacetic acid **(DOPAC)**, peak height (V) = $2.7 \times 10^{-4} \times$ (DOPAC (pg)) + 1.74×10^{-3} ($r^2 = 0.99$); and homovanillic acid **(HVA),** peak height (V) = $2.3 \times 10^{-4} \times$ (HVA (pg)) + 7.1×10^{-3} ($r^2 = 0.99$). Panel c, catecholamines norepinephrine **(NE),** peak height (V) = $4.06 \times 10^{-4} \times$ (NE (pg)) + 7.3×10^{-3} ($r^2 = 0.99$); and epinephrine **(E)**, peak height (V) = $2.43 \times 10^{-4} \times$ (E (pg)) + 7.6×10^{-3} ($r^2 = 0.99$).

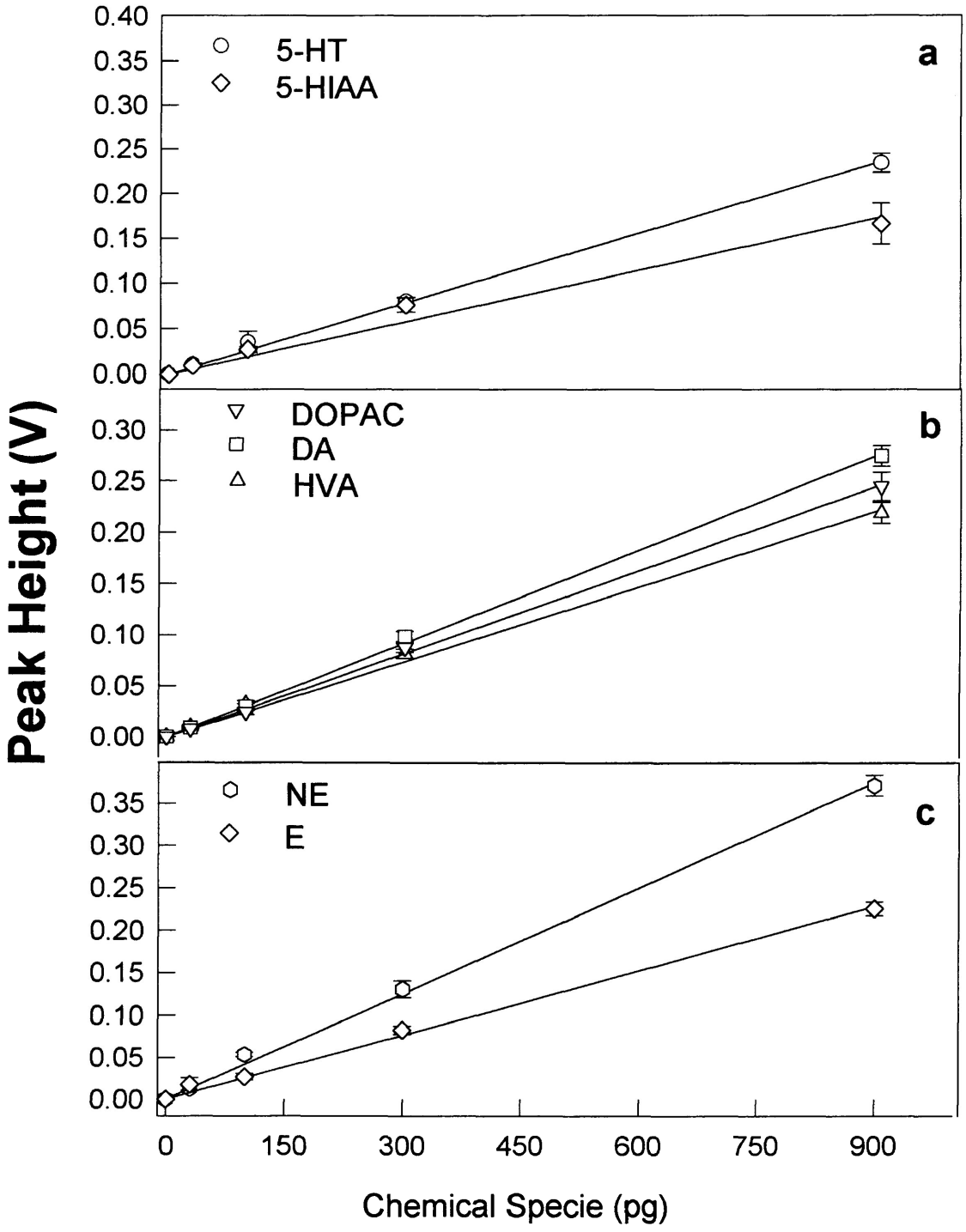
Peak Height (V)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
5-HT
5-HIAA
a
DOPAC
DA
HVA
b
NE
E
c
0
150
300
450
600
750
900
Chemical Specie (pg)

Fig. 3. Serotonin (5-HT, pmoles/g tissue) and specifically bound [^{3}H]ketanserin (B_{sp}, pmoles/g tissue) in selected brain regions of juvenile (clear bars) and sexually recrudescing female (shaded bars) rainbow trout. Panel A, 5-HT values are means (± SEM) from duplicate tissue samples from 6 independent experiments (n = 12 replicate determinations). 5-HT contents of tissue was statistically compared among tissues in the same age class of fish, on the basis of Kruskal Wallis test ($p<0.05$) and between juveniles and sexually recrudescing females in corresponding brain regions on the basis of Mann Whitney-U test ($p<0.05$). Data points which were not significantly ($p<0.05$) different share a common letter, case conserved. Panel B, Mean B_{sp} values (± SEM) from 4 independent experiments; in an individual experiment, quadruplicate determinations of B_o and NSB for a tissue preparation of pooled samples contributed to calculated mean values of B_o and NSB. $B_{sp\ MEAN}$ for a given experiment was the difference between $B_{o\ MEAN}$ and $NSB_{\ MEAN}$. Similarly, B_{sp} in tissues was statistically compared among tissues in the same age class of fish, on the basis of Kruskal Wallis test ($p<0.05$) and between juveniles and sexually recrudescing females in corresponding brain regions on the basis of Mann Whitney-U test ($p<0.05$). Data points which were not significantly ($p<0.05$) different share a common letter, case conserved.

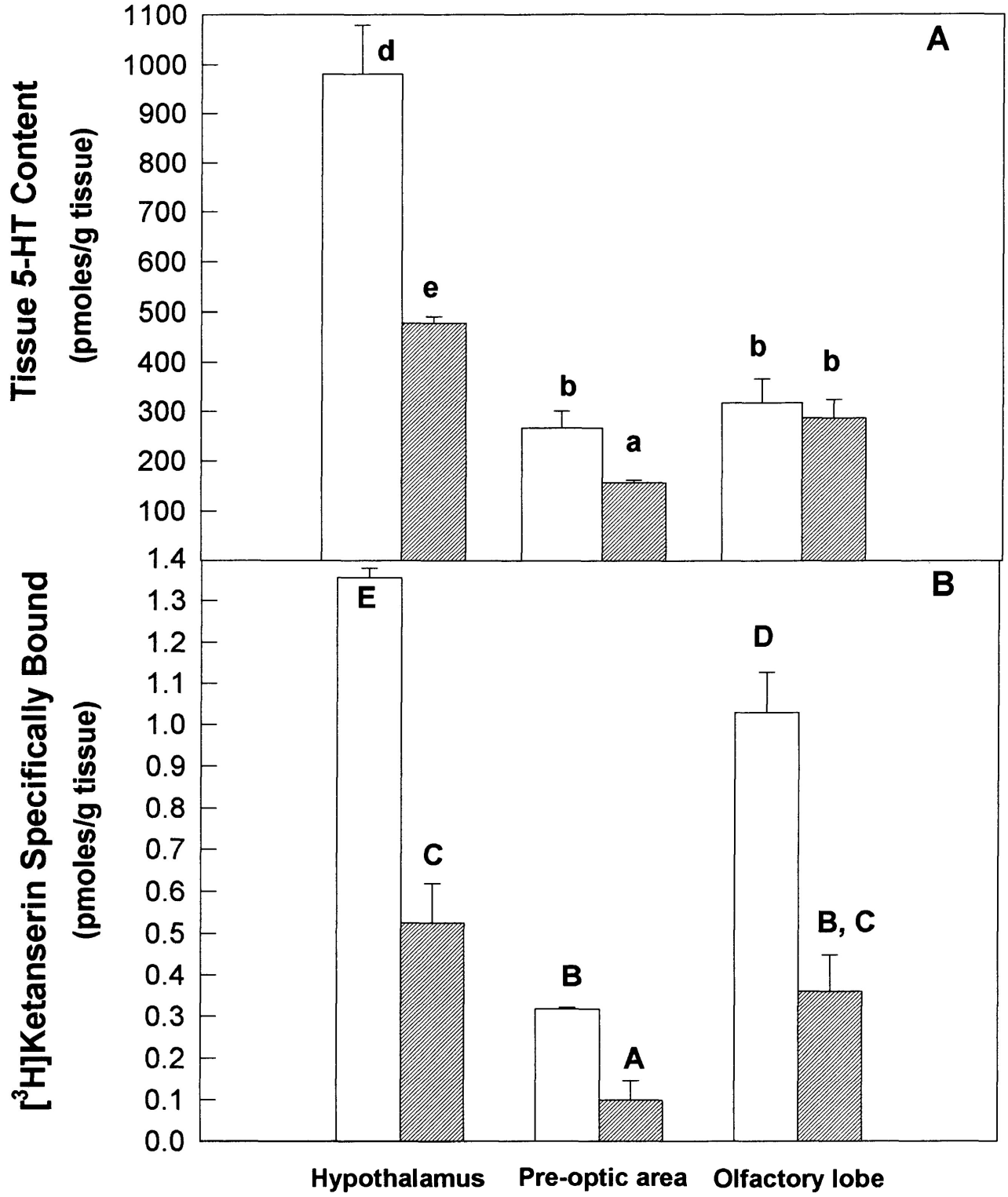
Tissue 5-HT Content
(pmoles/g tissue)
1100
1000
900
800
700
600
500
400
300
200
100
1.4
A
d
e
b
a
b
b
[3H]Ketanserin Specifically Bound
(pmoles/g tissue)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
B
E
C
B
A
D
B, C
Hypothalamus
Pre-optic area
Olfactory lobe

Fig. 4. [^{3}H]Ketanserin specifically bound (B_{sp}) (fmoles/g tissue) is plotted as a function of tissue 5-HT content (pmoles/g tissue). B_{sp} values were pooled from hypothalamus, pre-optic area, and olfactory lobe of juveniles and sexually recrudescing females. Linear regression of the data describes a relationship of [^{3}H]ketanserin specifically bound (fmoles/g tissue) = 1.43 x 5-HT (pmoles/g tissue) – 84.2 (r^2 = 0.994); [^{3}H]ketanserin specifically bound (fmoles/g tissue) = 1.3 x 5-HT (pmoles/g tissue) + 79 (r^2 = 0.7) if juvenile olfactory lobe data is <u>not</u> excluded.

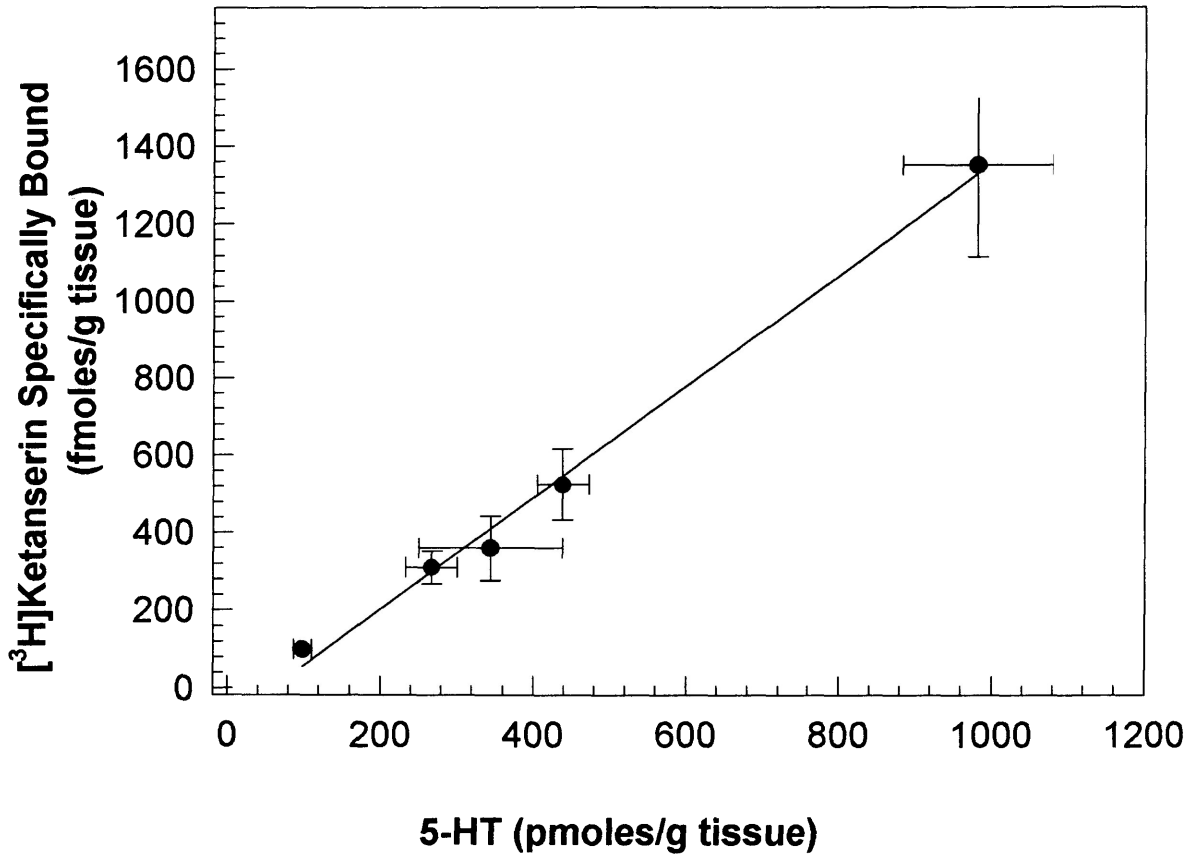
[3H]Ketanserin Specifically Bound
(fmoles/g tissue)
1600
1400
1200
1000
800
600
400
200
0
0
200
400
600
800
1000
1200
5-HT (pmoles/g tissue)

Table 1: Specifically bound [^{3}H]ketanserin in the brain regions of juvenile and sexually recrudescing female rainbow trout (*Oncorhynchus mykiss*)

	Specifically Bound [^{3}H]Ketanserin (cpm/mg protein)	
Tissue	**Juveniles**	**Sexually recrudescing females**
Olfactory lobe	987 (67) [c]	423 (34) [C]
Optic lobe	125 (23) [a]	64 (10) [A]
Pre-optic area	342 (58) [b]	65 (6) [A]
Hypothalamus	1620 (109) [d]	1100 (127) [D]
Spinal cord	285 (13) [a, b, *]	231 (29) [B, *]

Note: Values (cpm/mg protein) are means (± SEM), derived from 4 independent experiments each, with 4 replicates per experiment. Juvenile fish were approximately 24 months old and did not possess obvious gonads; sexually recrudescing females were approximately 36 months old and possessed obvious ovaries. B_{sp} in tissue types were statistically compared among tissues in the same age class by the Kruskal Wallis test; values which were not significantly different ($p<0.05$) share a common letter, case conserved. By comparison, Mann Whitney-U test was used to statistically compare B_{sp} levels between juveniles and females for a given tissue type. Values for specific tissue types between age classes which were not significantly different ($p<0.05$) are identified by an *.

Table 2: Retention times of standard biogenic amines and detected metabolites.

Specie	Retention time (min)
Norepinephrine	2.14 (0.021)
Epinephrine	2.36 (0.080)
DOPAC	3.00 (0.010)
Dopamine	3.14 (0.088)
5-HIAA	4.56 (0.210)
HVA	6.13 (0.240)
5-HT	7.27 (0.112)

Note: Values (min) are mean (± SEM) for species listed, from 8 independent experiments each, with 2 replicates per experiment.

Table 3: Biogenic amines and selected metabolites in brain regions of rainbow trout (*Oncorhynchus mykiss*).

Juveniles				
Species	**Olfactory lobes**	**Pre-optic area**	**Hypothalamus**	**Pituitary**
5-HT	78 (13) ^{a,*}	56 (7.3) ^{a}	228 (12) ^{b}	-
DA	753 (191) ^{c,*}	363 (24) ^{b}	1911 (143) ^{d}	6.1 (1) ^{a,*}
NE	1833 (128) ^{b}	940 (58) ^{a}	1699 (125) ^{b}	
HVA	97 (12) ^{b}		95 (2.1) ^{b}	5.2 (1) ^{a}
DOPAC				4.0 (1) ^{a,*}
5-HIAA		176 (27) ^{a}	183 (78) ^{a,*}	
Sexually recrudescing females				
5-HT	73 (20) ^{B,*}	21 (2.7) ^{A}	93 (7.3) ^{C}	-
DA	312 (74) ^{C,*}	170 (33) ^{B}	867 (47) ^{D}	5.0 (0.6) ^{A,*}
NE	1008 (56) ^{B}	507 (94) ^{A}	942 (80) ^{B}	
HVA		28 (4.4) ^{A}	53 (8.4) ^{A}	
DOPAC	80 (16) ^{B}			7.0 (1) ^{A,*}
5-HIAA	288 (27) ^{C}	88 (12) ^{A}	176 (10) ^{B,*}	

Note: Values (ng/g tissue) are means (n=6, $\pm$ SEM), from 6 independent experiments (in each experiment individual samples were analyzed twice by HPLC-EC analysis). Results from the pituitary are expressed as means ($\pm$ SEM, pg/μg of protein). Juvenile fish were 24 months old and did not possess obvious gonads, sexually recrudescing females were typically 36 months old and possessed obvious ovaries. – $\Rightarrow$ not detectable. Contents of specific chemical species were statistically compared (Kruskal Wallis test, $p<0.05$) among tissues in a given age class of animals; values which were not significantly ($p<0.05$) different share a common letter, case conserved. The contents of different chemical species within a single tissue type were not statistically compared. The content of a specific chemical specie in a given tissue type was statistically compared between age-classes on the basis of Mann Whitney-U test; values between age-classes that were not significantly ($p<0.05$) different share an asterix (*).

CHAPTER 4.

General Discussion

General Discussion

My results describe binding properties of [^{3}H]ketanserin to specific binding sites in trout hypothalamus (Chapter 2); the binding properties and ligand recognition criteria for these sites are comparable with those of the mammalian 5-HT_2 class of serotonin receptors. These 5-HT_2–like binding sites are differentially distributed among the brain regions examined. There is a very strong correlation of 5-HT_2-like binding site density with amounts of 5-HT and distribution of 5-HT (Chapter 3) in the brain regions of juvenile and sexually recrudescing female rainbow trout. In general, site density is typically greater in juveniles than in sexually recrudescent female trout. These findings support previous data on the distribution of 5-HT in teleost CNS and provide the first direct evidence for the existence, properties and distribution of a 5-HT_2-like binding site in trout brain and pituitary axis.

The presence of 5-HT cell bodies and serotonergic tracts have been previously reported in teleost brain:pituitary axis. To illustrate, large populations of 5-HT neurons are found in the midbrain, brain stem and diencephalon of goldfish (*Carassius auratus*; Kah and Chambolle 1983), African catfish (*Clarias gariepinus*; Corio et al. 1991), and rainbow trout (*Salmo gairdneri*; Frankenhuis-van den Heuvel and Nieuwenhuys 1984). Particularly high densities of 5-HT have been found in the nucleus raphe' medialis in various species of teleosts including the African catfish, Corio et al. 1991), three spined stickleback (*Gasterosteus aculeatus* L; Ekstrom and Van Veen 1984), sunfish (*Lepomis gibbosus*; Parent et al. 1978), and sockeye salmon (*Oncorhynchus nerka* Walbaum; Ekstrom and Ebbesson 1989). The raphe' nucleus projects large numbers of serotonergic axons to multiple regions of the teleost brain, including the hypothalamus, ventral thalamus, and

pituitary of the three spined stickleback (Ekstrom and Van Veen 1984), pars distalis of the pituitary gland, medulla oblongata, olfactory lobes, pre-optic area optic lobe and spinal cord of goldfish (Kah and Chambolle 1983), as well as the pre-optic nucleus in the African catfish (Corio et al. 1991).

Serotonin has prominent biological role(s) in these brain regions. For example, 5-HT regulates the secretion of growth hormone (GH) and gonadotropin (GtH) (Somoza and Peter 1991) from *in vitro* perifused goldfish pituitary fragments. 5-HT also stimulates release of maturational GtH from the pituitary of female Atlantic croaker (*Micropogonias undulatus*; Khan and Thomas 1992) and female and male goldfish (Somoza et al. 1988). In rainbow trout, Saligaut et al. (1992) demonstrated physiological fluctuations in hypothalamus and pituitary serotonin levels during ovarian recrudescence and ovulation; and Senthilkumar and Joy (1993) observed similar annual variations of serotonin levels in the hypothalamus of the Indian catfish (*Heteropneutes fossilis*). Although 5-HT pathways and biological functions are well studied in teleost brain regions, by contrast, there is little to no direct information on the existence of 5-HT receptors in teleost brain regions. However previous studies do indicate that 5-HT neurochemistry in teleost brain regions vary as a result of changes in sexual status. To illustrate, in 1-year old Atlantic croaker (*M.undulatus*, Khan and Thomas 1992), intraperitoneal administration of the combination of leutinizing hormone releasing hormone (LHRHa) and 5-HT elicited an increase in GtH levels, which were significantly greater than that induced by LHRHa or 5-HT alone. By comparison, 5-HT alone, stimulated GtH release both *in vitro* and *in vivo* using sexually mature croakers (Khan and Thomas 1992). These findings collectively suggest that 5-HT regulation may vary as a function of sexual status.

Specific binding of [3H]ketanserin to trout hypothalamus was saturable, indicating the presence of a finite number of binding sites in a definite region of brain tissue. The associable and reversible nature of this binding indicates that in accordance with ligand:receptor binding kinetics, [^{3}H]ketanserin associates and dissociates with the trout binding site in a predictable pattern consistent with a receptor. Differential displacement by various competitors, suggests that the binding site is specific (5-HT_2-like); it has differential binding affinity to various competitors depending upon their chemical structure. To illustrate, my results indicate that primarily ligands which are structurally related to ketanserin (4-substituted piperidine derivatives, 3-{2-[4 -4 fluorobenzoyl)-1-piperidinyl]ethyl-2,4 (1H, 3H)-quinazolinedione, Janssen 1983) or spiperone (8-[4 - (4 - fluorophenyl) 4-oxobutyl]-1-phenyl-1,3,8-triazaspirol [4,5]decan-4, Research Biochemicals International 1995) can successfully interact with the trout hypothalamic 5-HT_2-like binding site and implies a high degree of conservation of ligand recognition properties of this site.

The distributions of 5-HT and this binding site in teleost brain regions were predictable. To illustrate, in accordance with previous information on 5-HT neuron and fiber distribution as well as 5-HT role(s) in teleost brain regions, large amounts of 5-HT and its binding site were found in teleost hypothalamus compared to other brain regions. In my study, trout, (both juvenile and sexually recrudescing female), 5-HT concentration was greater in the hypothalamus (228 $\pm$ 12 ng/g tissue; 93 $\pm$ 7.3 ng/g tissue), than in both the pre-optic area (56 $\pm$ 7.3 ng/g tissue; 21 $\pm$ 3 ng/g tissue) and olfactory lobe (78 $\pm$ 13 ng/g tissue; 73 $\pm$ 20 ng/g tissue) (Table 3). In comparison, Saligaut et al. (1992) detected similar trends in 5-HT levels with higher levels of 5-HT (approximately 500 ng/g tissue) in

the hypothalamus of sexually recrudescing female rainbow trout, compared to the pre-optic area (approximately 150 ng/g tissue). Similarly, rank order of [^{3}H]ketanserin specific binding density in trout brain regions was hypothalamus > olfactory lobe >>> pre-optic area > spinal cord > optic lobe >>> pituitary. By comparison, in rats, the rank order of specifically bound [^{3}H]ketanserin density was prefrontal cortex (23.7 $\pm$ 0.5 pmoles/g tissue) >> temporal cortex (10.7 $\pm$ 2 pmoles/g tissue) >>>>>> hypothalamus (0.7 $\pm$ 0.4 pmoles/g tissue) >>>> pituitary (undetectable) (Leysen et al. 1982). Similar trends were found in guinea pig (Leysen et al. 1982 and Leysen et al. 1983) and human (Schotte et al. 1983) brain regions. Collectively, the distribution of 5-HT and its binding site in teleost brain regions were comparable with other vertebrate models.

Levels of both 5-HT and 5-HT$_2$ binding sites varied significantly among the olfactory lobe, optic lobe, pre-optic area and hypothalamus between juvenile and sexually recrudescing females, with levels in juveniles far exceeding those in brain regions of sexually recrudescing female trout. Although there is no direct previous information which compares levels of 5-HT and its binding site between juvenile and sexually recrudescing females in teleost models, indirect evidence suggest that sexual status influences 5-HT levels and functions in teleost brain:pituitary axis. For example, Saligaut et al (1992) observed an increase in 5-HT content in trout hypothalamus during the pre-ovulatory period, which provides direct evidence for changes in 5-HT synthesis and release influenced by sexual status in this region of the brain. This observation is consistent with changes in the distribution of 5-HT in the forebrain and pituitary observed in platyfish during reproductive senescence (Margolis-Nunno et al. 1986). These findings suggest that sexual status of the trout influences 5-HT and its binding site distribution in trout

brain:pituitary axis.

Collectively this research provides insight into potential 5-HT roles and function in the teleost brain:pituitary axis. 5-HT plays important role(s) in trout hypothalamus both prior to and at sexual maturity, therefore the highest observed levels of both 5-HT and specific binding site 5-HT_2, are in the hypothalamus. 5-HT plays important roles in trout sexual maturation and growth; this observation is consistent with my finding of difference in levels of 5-HT and its binding sites (5-HT_2) between juveniles and sexually recrudescing females. 5-HT role(s) differ between brain regions in the same class of trout; to illustrate, differing 5-HT:B_{sp} ratios between the olfactory lobe and other brain regions suggest that 5-HT neural activity in the olfactory lobe differs from that in other elements of the pre-optic-hypothalamo-hypophysial axis. These findings provide fertile ground for future projects involving direct neuroendocrine role(s) of 5-HT and its binding site in the teleost brain:pituitary axis.

Therefore to conclude, my results demonstrate the presence of both, 5-HT and its specific 5-HT_2-like binding site in the brain:pituitary regions of the rainbow trout. Levels of both 5-HT and its binding site differ between specific brain regions, with highest levels in the hypothalamus. Also, levels of both 5-HT and its binding site differ between juvenile and sexually recrudescing female trout, suggesting that 5-HT role(s), prominent in teleost hypothalamus are influenced by the sexual status of the fish. Differing 5-HT:B_{sp} ratio in the olfactory lobe suggest that 5-HT regulation of the olfactory lobe differs from that in the pre-optic-hypothalamo-hypophysial axis. This demonstration of the existence of 5-HT and its binding site in trout brain regions, in my study, is a timely contribution to the already existent information on 5-HT role(s) in teleost brain:pituitary axis.

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APPENDICES

Appendix 1

Determination of kinetically derived estimates of k_{-1}, k_{+1}, and K_D.

The kinetically derived equilibrium dissociation constant (K_D) was determined on the basis of association and dissociation experiments; $K_D = k_{-1}/k_{+1}$ where k_{-1} and k_{+1} represent the rate of dissociation and the rate of association respectively.

k_{-1} was based on a semilogarithmic plot of dissociation data (Fig 2b, chapter 2) wherein, ln B_{sp}/B_{zerot} was plotted as a function of time t (min); where B_{zerot} was B_{sp} immediately before addition of 5000 fold excess unlabelled ketanserin. The equation of the line was ln B/B_{zerot} = 0.0803 x time + 0.417 , $r^2 = 0.93$, with slope of the line (k_{-1}) = 0.0803 min^{-1}, and half –life ($t_{1/2}$) = ln (0.5) /k_{-1} = 8.7 min (Bylund and Yamamura, 1990).

k_{+1} estimated from a pseudo first-order association plot (Fig 2a, chapter 2), was based on equation of the line [ln(Be/Be-B)] = 0.129 x time + 0.0437 , r^2=0.9 (Bylund and Yamamura, 1990), the slope of which (k_{obs}) was 0.129 min^{-1}.

$k_{+1} = (k_{obs} - k_{-1})/F$, where F is the concentration of free radioligand.

$$k_{+1} \qquad (k_{obs} - k_{-1}) / F$$

$$\frac{0.129-0.0803}{1nM}$$

$$0.048\ min^{-1}\ nM^{-1}$$

K_D for [^{3}H]ketanserin binding to hypothalamic membrane preparation was calculated as:

$$K_D \qquad k_{-1}\ (min^{-1})/k_{+1}\ (min^{-1}\ nM^{-1})$$

$$0.0803/0.048$$

$$1.67\ nM.$$

Appendix 2

Determination of Scatchard estimated values of K_D.

In a Scatchard analysis (Scatchard 1949), the ratio of bound to free (B/F) radioligand is plotted versus bound radioligand (B). The equilibrium dissociation constant (K_D) is the negative reciprocal of the slope, and maximum binding capacity (B_{MAX}; 250 fmol/mg protein) is the intercept on the x-axis. For saturation experiments (Fig 3, inset, Chapter 2), K_D and B_{MAX} were estimated from the equation of the straight-line: (Bound/Free = -2.07×10^9 Bound + 0.03, r^2=0.95).

Therefore: K_D $(-\text{ slope})^{-1}$ nM^{-1}

$-(-2.07)^{-1}$ nM^{-1}

0.48 nM

Similarly for displacement data, estimates of inhibition constant (K_i; affinity of the inhibitor for the 5-HT_2-like binding site, Table 1, Chapter 2), for each competitor were based on Scatchard analysis (1946) where the ratio of bound to free (B/F) radioligand is plotted versus bound radioligand (B).

Half-maximal inhibitory concentration (IC_{50}) values for each competitor were estimated from logit-log plots: logit values (logit = ln[P/(100 –P)], P is percent bound) of total [^{3}H]ketanserin binding to trout hypothalamic membrane preparation versus -log [competitor, M] (Bylund and Yamamura 1990). The IC_{50} value was the concentration of competitor when P=50% (Table 1, Chapter 2).

logit ln [P/(100-P)]; where

P $\frac{B - NSB}{B_o} \times 100$

Where B_o amount of B_{sp} in the absence of competing drug.

The accuracy of K_i values from Scatchard analysis (1946) was confirmed by comparable K_i values, estimated by the Cheng & Prusoff (1973) equation,

$K_i = IC_{50} / [1 + C / K_D]$, where C is concentration of radioligand and K_D is dissociation rate constant obtained from saturation experiments.

Appendix 3

1. Estimation of specifically bound [^{3}H]ketanserin B_{sp} (cpm) as pmoles/g tissue (wet weight). (All conversions for binding data were based on this calculation).

Radioactivity of sample	"a" cpm
Conversion of cpm to dpm (50% efficiency)	a cpm ÷ (0.5 cpm/dpm) = 2a dpm
Conversion of dpm to Ci	2a dpm ÷ 2.2×10^{12} dpm/Ci $= 0.909a \times 10^{-12}$ Ci
Conversion of Ci to moles (S.A = 61 * 10^3 Ci/mol)	$0.909a \times 10^{-12}$ Ci ÷ 61×10^3 Ci/mol $= 0.149a \times 10^{-15}$ mol
Relation of moles to tissue mass	$= 0.149a \times 10^{-15}$ mol ÷ "y" tissue weight (g) $= \frac{0.149a \times 10^{-15} \text{ x}}{\text{y}}$ (mol/g tissue)

2. The equation of straight line from standard graphs of biogenic amines and metabolites were used to estimate pg as pmoles/g tissue (wet weight) in HPLC-EC analysis. Specie concentration (pg) was calculated from corresponding peak height (volts) values.

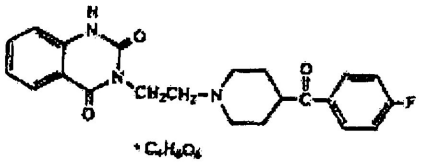
Level of chemical specie detected	"b" $\times 10^{-12}$ g
Total amount in 1 tissue (1 tissue was homogenized in 500 µl $HClO_4$ each (except pituitary in 200 µl $HClO_4$), but injection volume was 10 µl)	("b" $\times 10^{-12}$ g)x 50
Conversion of pg to moles	$50b \times 10^{-12}$ g ÷ MW (g/mol) $= 50b \times 10^{-12}$ mol
Relation of moles to tissue mass	$50b \times 10^{-12}$ moles ÷ "y" tissue (g) $= \frac{50b \times 10^{-12} \text{x}}{\text{y}}$ (mol/g tissue)

Appendix 4

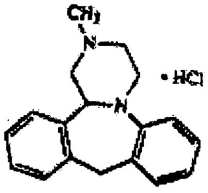
1. Chemical structures of competitors used in displacement experiments (Chapter 2). Figures adapted from Research Biochemicals International 1995Catalog/ Handbook.

Group I

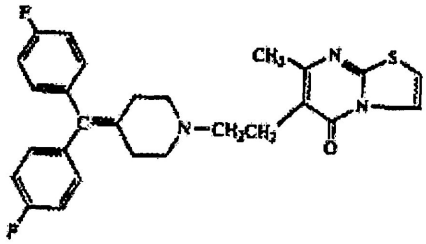
Ketanserin



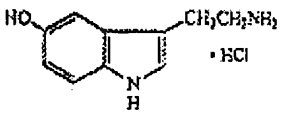
Mianserin



8 Ritanserin
9

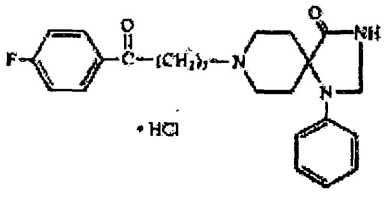


Serotonin

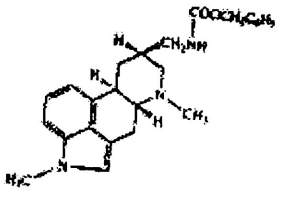


1 Group II

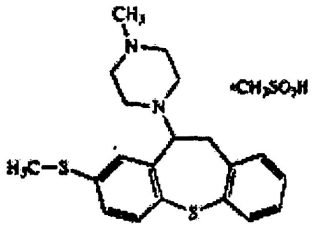
2 Spiperone



Metergoline

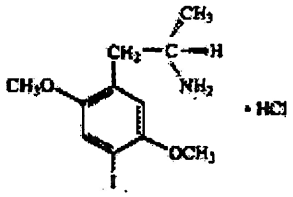


Methiothepin mesylate

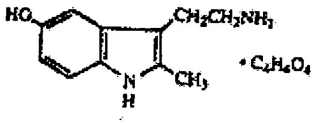


Group III

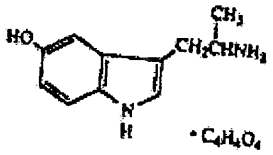
DOI



2-methyl-5-HT-maleate

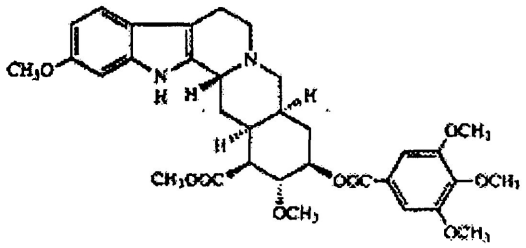


α-methyl-5-HT-maleate

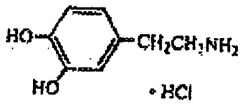


Group IV

Reserpine



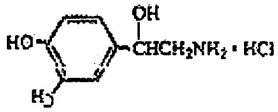
5-HIAA



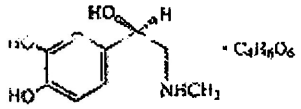
2. Chemical structures of biogenic amines used in HPLC-EC analysis (Chapter 3).

Group I (Biogenic amines)

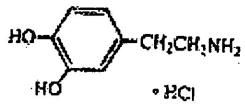
Norepinephrine



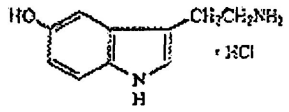
Epinephrine



Dopamine

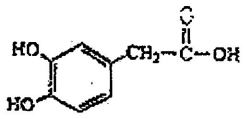


Serotonin

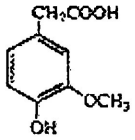


Group II (Metabolites)

DOPAC



6
7
8 HVA



5-HIAA