THYROTROPIN RELEASING HORMONE (TRH) RECEPTORS IN THE HYPOTHALAMUS AND PITUITARY OF RAINBOW TROUT (*Oncorhynchus mykiss*) AND PITUITARY OF GOLDFISH (*Carassius auratus*)

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ABSTRACT

Thyrotropin releasing hormone (TRH; pGlu-His-Pro-NH_2) binding sites in the rainbow trout (*Oncorhynchus mykiss*) pituitary and hypothalamus, and receptors in the goldfish (*Carassius auratus*) pituitary, were examined by radioreceptor assay (RRA) using the TRH analog [^{3}H](3-Me-His^2)TRH (MeTRH) as a radioligand. [^{3}H]MeTRH binding was displaceable from trout pituitary membrane preparation; LIGAND-analysis of homologous (MeTRH displacement of [^{3}H]MeTRH) displacement experiments estimate the dissociation rate constant (K_d) and capacity (B_{max}) as 6.93 X 10^{-9} M and 530 X 10^{-15} mol/mg protein respectively. Heterologous (TRH) displacement of [^{3}H]MeTRH from whole trout pituitary homogenate demonstrated two classes of TRH receptors; K_d's were estimated to be 4.15 X 10^{-12} M and 3.40 X 10^{-8} M with B_{max}= 147.68 and 458.85 X 10^{-15} mol/mg protein respectively. These two receptor classes appeared to be segregated between the neurointermediate lobe (NIL) and pars distalis (PD), where the K_d and B_{max} for the NIL sites were 5.62 X 10^{-9} M and 252.64 X 10^{-15} mol/mg protein and 4.21 X 10^{-11} M and 87.41 X 10^{-15} mol/mg protein for the PD sites. The goldfish pituitary, by comparison, contained two populations of TRH binding sites segregated between the NIL and PD with estimated K_d and B_{max} of 2.18 X 10^{-9} M and 320.25 X 10^{-15} mol/mg protein respectively for the NIL and 8.77 X 10^{-12} M and 84.05 X 10^{-15} mol/mg protein for the PD. Homologous displacement of [^{3}H]MeTRH from trout hypothalamus preparation revealed a single class of TRH binding-sites with estimated K_d = 6.91 X 10^{-9} M and B_{max} = 8.84 X 10^{-15} mol/mg protein. Native TRH also dose-dependently displaced radioligand from hypothalamus membrane preparation; LIGAND-estimates for K_d and B_{max} were 1.52 X 10^{-9} M and 3.79 X 10^{-15} mol/mg protein respectively.

The structural requirements of TRH stimulation of α-melanocyte stimulating hormone (α-MSH) release from superfused trout pituitary fragments were also investigated. Trout pituitaries were separated into individual lobes, fragmented, pooled and superfused with acute doses of TRH or TRH-analog 10 minute fractions were collected and α-MSH content was later accessed by specific RIA. Native TRH stimulated acute increases in α-MSH release with an estimated ED_{50} of 1.73 X 10^{-9} M on the basis of increasing-dose response data and estimated ED_{50} of 1.57 X 10^{-9} M on the basis of decreasing-dose response data. The minimum effective dose for TRH stimulation of α-MSH release from the trout NIL fragments was 10^{-9} M in both increasing- and decreasing-dose repsponse experiments. Repeated TRH doses at high concentration (10^{-6} M) consistantly produced similar α-MSH release responses, suggesting no up- or down-regulatory effects on α-MSH release. Of the analogs examined, MeTRH was more potent than the native ligand (minimum effective dose: 10^{-10} M and ED_{50}: 1.56 X 10^{-9} M); substitution at the central histidine residue ([Phe^2]TRH) produces an analog with minimal bioactivity (minimum effective dose = 10^{-6} M) and substitution at either the amino terminus ([Glu^1]TRH or [1-Me-S-dihydrooratyl]TRH) or carboxy terminus (pGlu-His or TRH-Gly) resulted in a near complete loss of bioactivity.

This study indicates the presence and binding characteristics of specific TRH-binding sites in the salmonid hypothalamus and pituitary as well as the cyprinid pituitary; furthermore, this study demonstrates the strict structural requirements of the trout pituitary TRH receptor with respect to the TRH induced stimulation of α-MSH release from the trout NIL and provides the first evidence of direct TRH stimulation of salmonid melanotropes.

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INTRODUCTION

Thyrotropin releasing hormone (TRH) (L-(pyro)Glu-His-Pro-NH_2; Boler *et al.*, 1969; Folkers *et al.*, 1970) is a phylogenetically conserved neurohormone (Jackson, 1986) and was initially found to stimulate the release of thyroid stimulating hormone (TSH) from porcine pituitary glands (Schally *et al.*, 1969). In other mammals, including man, TRH stimulates the release of TSH and prolactin (PRL) from the pituitary (Jackson, 1981). The actions of TRH in fishes are controversial; for example, the TSH-releasing activity of TRH remains unresolved. Examination of TRH effects on the release of thyroid hormones in fishes has brought forth conflicting evidence showing signs of both increased (Eales and Himick, 1988) and decreased (Bromage, 1975) thyroidal activity. By comparison, TRH stimulates the release of alpha-melanocyte stimulating hormone (α-MSH) (Omeljaniuk *et al.*, 1989; Tran *et al.*, 1989) and adrenocorticotrophic hormone (ACTH) (Tran *et al.*, 1989); both characteristic stress response hormones. Evidence also suggests that TRH stimulates the release of PRL and growth hormone (GH) from the sailfin molly pituitary (Wigham and Batten, 1984).

Despite the growing evidence of TRH bioactivity in the teleost pituitary there is little information on teleost pituitary TRH-receptors. Burt and Ajah (1984) have reported TRH-receptors located in the goldfish brain with a cursory examination of the pituitary receptors from a variety of pooled marine fishes.

In this study, the existence and binding parameters of TRH receptors in the rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*) pituitary were investigated. The structural requirements for TRH-induced stimulation of α-MSH release

from the trout neurointermediate lobe (NIL) were also examined using various TRH analogs. The hypothalamus, site of neurohormonal regulation of pituitary function at the pre-hypophyseal level, was also examined for specific TRH receptors.

LITERATURE REVIEW

TRH IN FISH:

DISTRIBUTION:

In teleosts, TRH generally appears to be produced in the hypothalamic nuclei and carried to the NIL via neurosecretory fibres to make direct contact or terminate in close proximity to the target cells. Teleost fishes lack a functional hypothalamo-hypophyseal blood portal system (Ball, 1981) as found in higher vertebrates therefore, TRH induced secretion of other hormones must be a result of direct or proximal innervation of the target cell types, as in the case of the melanotrophs of the NIL, or via TRH circulation throughout the systemic system. It is possible that the circulating TRH may stimulate the release of the other hormones since there appears to be no direct innervation in the PD as determined by Batten *et al.* (1990); however, a more likely possibility exists where TRH acting on the PD is supplied from the NIL.

Numerous immunoreactive TRH (ir-TRH) nerve fibres are found in the carp (*Cyprinus carpio*) hypothalamus, extending from the preoptic nucleus (NPO) to the nucleus recessus lateralis (NRL) where the cell bodies originate (Hamano *et al.*, 1990). TRH fibres have been demonstrated in the neurointermediate lobe (NIL) of the carp pituitary but not in the anterior pituitary (AP) (Hamano *et al.*, 1990) suggesting a lack of direct contact between the hypothalamus and the AP. This lack of contact between the hypothalamus and pars distalis (PD) poses interesting questions with respect to the demonstrated TRH stimulation of gowth hormone (GH) and prolactin (PRL) secretion (Wigham and Batten, 1984). There is no information available regarding the distribution

of TRH in the brain or pituitary gland of salmonids.

In the sea bass (*Dicentrarchus labrax*), ir-TRH fibres are distributed throughout the NIL in close proximity to the melanotrophs (Batten *et al.*, 1990), suggesting a neuroendocrine role. ir-TRH fibres have not been found in the sea bass pars distalis (PD) where GH and TSH cells are located nor are immunoreactive fibres located in the rostral PD where the PRL and ACTH secreting cells are located (Batten *et al.*, 1990). TRH is also distributed throughout the CNS of the african lungfish (*Protpoterus annectens*) with high concentrations in the telencephalon and diencephalon (Kreider *et al.*, 1988) suggesting a role as a neurotransmitter.

ACTIONS:

The actions of TRH in fish have been controversial. Gorbman and Hyder (1972) demonstrated that injection of TRH at doses up to 2 µg/g body weight had little effect on thyroidal ^{131}I uptake in the african lungfish (*Protopterus ethiopicus*) suggesting TRH does not effect thyroid hormone synthesis either directly or via induced thyroid stimulating hormone (TSH) activity. In mammals, TRH at nanogram concentrations increases thyroidal radioiodine uptake. These results suggest that in fishes, TRH does not alter thyroid function either directly or through modification of TSH in fish as it does in the mammals. Bromage (1975) indicated TRH may decrease thyroidal activity in the guppy (*Poecilia reticulata*) since histochemical examination of the thyroid follicles showed increased thyroid colloid density consistant with signs of decreased activity following TRH injection. Co-culturing thyroid cells with pituitary cells from the pacific hagfish (*Eptatretus stouti*) resulted in increased thyroxine (T_4) secretion suggesting pituitary

regulation of thyroid activity. By contrast, incubation with medium containing 50 ng TRH/ml of culture medium failed to significantly increase the medium T_4 concentrations (Dickhoff *et al.*, 1978). The apparant inability of TRH to increase thyroidal activity in this model was attributed to the lack of TSH-releasing activity from the teleost pituitary as compared to mammals, where TRH stimulates the release of TSH which in turn increases the thyroidal release of T_4. By comparison, Eales and Himick (1988) demonstrated increased plasma T_4 levels in the rainbow trout (*Oncorhynchus mykiss*) and the arctic charr (*Salvelinus alpinus*) following a single intraperitoneal injection of TRH (1 μg/g). It should be noted that the plasma levels of triiodothyronine (T_3) and T_4 increase as a result of physical injury to *Oncorhynchus mykiss* as a result of injection or blood extraction within two hours of the trauma (Brown *et al.*, 1978). Therefore extreme care must be exercised when evaluating the effects of TRH on systems of this nature. These data also suggest that regulation of thyroid function is a sensitive indicator of stress response.

TRH concentrations in the diencephalon of african lungfish (*Protopterus annectens*) decrease during estivation (Kreider *et al.*, 1990). These reductions parallel the decreased TRH concentrations in the hypothalamus of the ground squirrel (*Citellus lateralis*) during its hibernation (Stanton *et al.*, 1992). Starvation has no effect on TRH concentrations in the lungfish CNS (Kreider *et al.*, 1990). These data again lend support to a possible TRH role in the regulation of teleost arousal state as well as stress-response modulation.

Recently, TRH bioactivity in the teleost pituitary has been investigated using the

goldfish. TRH has been shown to stimulate the release of α-MSH from goldfish NIL fragments *in vitro* (Omeljaniuk *et al.*, 1989). Acute α-MSH release responses to brief TRH pulses suggest a direct and reversible TRH action; the molecular specificity of TRH stimulation in the goldfish and rainbow trout is not known. TRH stimulated α-MSH release from goldfish NIL fragments is significantly inhibited by dopamine (DA) and the DA agonists (-)-apomorphine in a dose-related manner (Omeljaniuk *et al.*, 1989). Moreover, domperidone, a specific DA:D2 receptor antagonist, blocks the inhibitory DA effect and reinstates TRH stimulated release of α-MSH from the goldfish NIL (Omeljaniuk *et al.*, 1989). Dopaminergic inhibition of α-MSH release may be partially responsible for the large releases of α-MSH from freshly isolated goldfish pituitary fragments, recently disconnected from dopaminergic inhibition. *In vivo*, pimozide, sulpiride, and domperidone (dopamine antagonists) induce morphological changes in goldfish NIL melanotropes (Olivereau *et al.*, 1987). The potential role of DA inhibition of α-MSH release bears striking similarity to DA inhibition of gonadotropin release and GnRH action in goldfish (Omeljaniuk *et al.*, 1989). This parallelism suggests a close relationship between regulation of reproductive and stress response hormone release from the pituitary.

A further study investigated TRH-stimulation of dispersed goldfish NIL cells during *in vitro* perfusion results in dose-dependent releases of α-MSH and ACTH with maximal stimulation occurring at 50 nM and 5 nM respectively (Tran *et al.*, 1989). These data support the concept that TRH may be a stress response neurohormone which may complement the actions of corticotropin releasing factor (CRF), a recognized stress

response neurohormone, in the regulation of ACTH release. ACTH release is also actively stimulated from dispersed goldfish PD cells by angiotensins I and II (Weld and Fryer, 1987). Urotensin I, a CRF-like peptide, is also effective in stimulating the *in vivo* release of ACTH in the white sucker (*Catostomus commersoni*); ACTH release is also stimulated from superfused PD cells *in vitro* (Fryer *et al.*, 1983). There are thus far no reports on the co-ordinated actions of TRH and other regulatory factors in the regulation of ACTH release. Sumpter (1986) determined that several forms of immunoreactive ACTH (ir-ACTH) could be detected in the trout NIL and PD with the PD containing approximately 3 times as much as the NIL. Interestingly, none of the ir-ACTH forms found in the PD were found in the NIL and vice versa (Sumpter, 1986). Acute stress of mammals increases ACTH release; similarly, handling and confinement stresses in salmonids significantly increase ACTH release while having no effect on α-MSH release (Sumpter and Donaldson, 1986). More severe stress, removal from water for five minutes followed by 25 minutes confinement in a small volume of water, resulted in ACTH levels rising then falling during the 25 minute confinement; α-MSH levels also increased significantly following this stress (Sumpter *et al.*, 1986).

TRH further functions to stimulate growth hormone (GH) release from goldfish PD fragments *in vitro* (Trudeau *et al.*, 1992). Although no clear dose-response relationship was evident, TRH (10 to 10 000 nM) consistently stimulated increased GH release from superfused PD fragments (Trudeau *et al.*, 1992). Significant increases in GH secretion from cultured pituitary tissue of the sailfin molly (*Poecilia latipinna*) were detected following culture in medium containing 28 μM TRH (Wigham and Batten,

1984). The role of TRH in regulating PRL release from the teleost PD remains unclear with some evidence suggesting a stimulatory role while other evidence suggests an inhibitory role. Incubation in hyperosmotic medium (340 mosmol/kg) increased PRL secretion from the pituitary cells of the sailfin molly; however, incubation in 300 mosmol/kg medium displays no detectable stimulation of PRL in the presence of TRH (Wigham and Batten, 1984). By comparison, TRH (28 μM) inhibited PRL release from tilapia (*Sarotherodon mossambicus*) PD fragments incubated in a hypoosmotic medium while having no effect in hyperosmotic media (Wigham *et al.*, 1977). Together, these findings suggest that osmotic pressure imparts some degree of regulation on PRL synthesis and secretion in teleosts. However, the osmolality of the corresponding media was adjusted by alterations in NaCl concentration (Wigham *et al.*, 1977; Wigham and Batten, 1984) suggesting possible changes in membrane voltage associated with PRL secretion. TRH binding to GH cells is inhibited by monovalent cations (half-maximal inhibition (IC_{50}) = 30 mM) (Hinkle and Kinsella, 1984).

The mechanisms of TRH action in teleosts are as yet unclear; however, in the ventral lobe of the dogfish (*Scyliorhinus canicula*) pituitary, TRH increases adenylyl cyclase activity (Deery and Jones, 1975) suggesting cAMP moderated actions. Adenylyl cyclase converts adenosine monophosphate (AMP) to cyclic-adenosine monophosphate (cAMP), an intracellular second messenger. As the ventral lobe of the dogfish pituitary does not appear to be innervated, hormonal regulation of this system may be subject to circulating hormones and neurohormones. There appears to be no other record of TRH signalling mechanisms in teleosts.

RECEPTORS:

Very little work has been done with respect to teleost pituitary TRH receptors in teleosts. Burt and Ajah (1984) described a TRH radioreceptor assay (RRA) for the fish brain, pituitary, and spinal cord; however, there was little data on the goldfish pituitary TRH receptor. In the brain two types of TRH binding sites were demonstrated, with either high affinity or low affinity (Burt and Ajah, 1984). The high-affinity sites on the goldfish brain have a dissociation constant (K_D) of 3.74 nM and a capacity (B_{max}) of 148 pM/mg protein (Burt and Ajah, 1984). The existence of the second class of low-affinity binding sites (K_d approximately 15 μM; Burt and Ajah, 1984) opens an interesting avenue for future research since the apparent receptor affinity suggests possible TRH function in the low micromolar range. There is little information on the specificity or regional distribution of TRH receptors in teleosts; although large concentrations of TRH have been characterized in the telencephalon and diencephalon of the african lungfish (*Protopterus annectens*) with smaller concentrations in the medulla (Kreider, *et al*, 1988).

TRH IN MAMMALS:

DISTRIBUTION:

Jackson and Reichlin (1974) conducted an in-depth study of TRH distribution in mammalian and non-mammalian vertebrates where they found large concentrations of TRH in the hypothalamus of the rat (*Rattus norvegicus*), frog (*Rana pipiens*), chicken (*Gallus domesticus*), and salmon (*Salmo sebago*). The high concentrations of TRH in the rat hypothalamus is consistent with its role in the regulation of mammalian TSH release from the anterior pituitary. In mammals, TRH is produced in the hypothalamus and

transported to the pituitary where TSH release is stimulated. Small concentrations of TRH were found in the rat cerebellum, cerebral cortex, brain stem and olfactory lobe (Jackson and Reichlin, 1974). In comparison, larger concentrations of TRH were found in homologous regions of the brain of the frog and salmon (Jackson and Reichlin, 1974).

TRH is distributed throughout the neuronal processes of the mammalian hypothalamus. The densest concentration of ir-TRH is found in the rostral-caudal portion of the median eminence and the pituitary-stalk/median eminence in close proximity to the portal capillaries leading to the anterior pituitary (Lechan and Jackson, 1982). Other hypothalamic regions containing large amounts of TRH include the paraventricular nucleus, arcuate nucleus, perifornical region and the periventricular nucleus (Lechan and Jackson, 1982). Smaller amounts of ir-TRH can be detected throughout the remaining regions of the hypothalamus (Lechan and Jackson, 1982).

The posterior pituitary (neural lobe) contains a large number of ir-TRH fibres originating in the hypothalamus while the intermediate lobe and the anterior pituitary have none (Lechan and Jackson, 1982). While the mammalian pituitary comprises three distinct regions (neural lobe, intermediate lobe, and pars distalis), the teleost pituitary differs in that the neural and intermediate lobes are integrated forming the neurointermediate lobe (NIL). Specific TRH receptors have been characterized in the pituitaries of the rat (Burt and Snyder, 1975; Sharif *et al.*, 1991), sheep (Burt and Taylor, 1980; Sharif *et al.*, 1991), dog (Sharif *et al.*, 1991), and cow (Sharif *et al.*, 1991). TRH has not only been isolated in the brain and pituitary regions of the rat but is also found distributed throughout the nervous system and other organ systems such as the stomach

of the gastrointestinal tract where it functions as a neurotransmitter (Jackson, 1986).

ACTIONS:

The small size and rapid rate of deactivation, most commonly through deamidation (Lechan and Jackson, 1982), are properties consistent with a neurotransmitter. It is possible that TRH evolved into its present role as a releasing agent from a more primitive role as a neurotransmitter or neuromodulator in the CNS. The ability of TRH, in doses as low as 1.0 ng, to stimulate the release of TSH from the rat anterior pituitary has been well documented (Martin and Reichlin, 1972), though TRH is somewhat less effective than MeTRH in stimulating TSH release (Dannies and Markell, 1980).

TRH significantly increases PRL release from rat anterior pituitary cells (Hyde and Keller, 1991) as well as cultured GH cells (Tsai and Samuels, 1974). Again, MeTRH proves to be more effective than TRH in stimulating the release of PRL in the rat (Dannies and Markell, 1980). Hyde and Keller (1991) also demonstrated that TRH is effective in stimulating the secretion of galanin from the rat anterior pituitary. Dopamine and somatostatin both inhibit the release of PRL and galanin from the anterior pituitary and thus may possibly have an effect on the release or the receptor binding of TRH (Hyde and Keller, 1991).

Starvation significantly increases TRH receptors in the rat corresponding to the degree of food deprivation, suggesting relevance to the mammalian response to starvation (Rodriguez *et al.*, 1991). In comparison, a previous study failed to show any significant increases in either the affinity or number of TRH receptors in starved as opposed to fed rats (Haugues *et al.*, 1987).

RECEPTOR MODULATION:

[^{3}H]TRH binds to a plasma membrane fraction of bovine pituitary glands in a time dependent manner and is displaced by addition of unlabeled TRH (Labrie *et al.*, 1972). Binding of radiolabeled TRH was not affected by the presence of GH, PRL, ACTH, T_3, or T_4. K^+ or Mg^{++} increased receptor binding at low concentrations but were inhibitory at higher concentrations (Labrie, *et al.*, 1972). By comparison, Ca^{++} ions inhibited TRH binding at low concentrations, suggesting the involvement of Ca^{++} ions in the TRH signalling mechanism in the bovine pituitary (Labrie *et al.*, 1972). TRH binding was also inhibited by monovalent cations such as Na^+ and Li^+ with half-maximal inhibition occurring at 30 mM concentrations (Hinkle and Kinsella, 1984). The cation inhibition may occur at the receptor site since both the number and the affinity of the receptor sites decrease and the dissociation rate increases (Hinkle and Kinsella, 1984).

Pituitary TRH receptors in the rat are subject to both homologous and heterologous regulation (Hinkle, 1989). Homologous down-regulation results from the binding of TRH to its receptor leading to a decrease in the number of available binding sites for the hormone (Hinkle, 1989). Heterologous down-regulation, regulation of the TRH receptors via a different hormone or drug, may be direct or indirect (Hinkle, 1989); for example, thyroid hormones decrease TRH receptor numbers (Hinkle, 1989) while estrogens (DeLean *et al.*, 1977) and glucocorticoids (Hinkle, 1989) increase pituitary TRH receptor levels through an unknown mechanism. Drugs leading to elevated levels of cAMP (eg. isobutylmethylxanthine and cholera toxin) reduce the number of TRH receptors while activators of protein kinase C, such as phorbol esters, heterologously decrease receptor

affinity (Hinkle, 1989). Cholera toxin also decreases [^{3}H]methyl-TRH binding to cultured GH_3 receptors following incubation at 50 ng/ml (Yajima *et al.*, 1988); supporting the concept of TRH receptors linking with G-proteins in mammals. The putative G-protein, coupled to phospholipase C (Bauer *et al.*, 1990), is thought to gate the TRH-receptor mediated breakdown of phosphatidylinositol-4,5-bisphosphate to the second messengers: 1,3,4-inositol-triphosphate (IP_3) and diacylglycerol (DAG) (Phillips and Hinkle, 1988). IP_3 leads to the mobilization of intracellular calcium while DAG activates protein kinase C, resulting in the release of the TRH-stimulated hormone (Phillips and Hinkle, 1988).

TRH IN AMPHIBIANS, BIRDS AND REPTILES:

TRH has been demonstrated in large concentrations in the epidermal epithelium (Jackson and Reichlin, 1977) and blood (Jackson and Reichlin, 1979) of the frog (*Rana pipiens*). Jackson and Reichlin (1977) proposed that the circulating TRH was somehow derived from the epidermis. TRH release from the epidermis appears to be under noradrenergic (stimulatory) and dopaminergic (inhibitory) control since incubation of the skin with 10^{-4} M norepinephrine (NE) consistently stimulated TRH release while incubation with DA at concentrations as low as 10^{-12} M inhibited TRH release from the skin in a dose-related manner (Bolaffi and Jackson, 1982). Intravenous injections of low doses (1 μg) into the frog (*Rana ridibunda*), increased circulating T_3 and T_4 levels (Darras and Kuhn, 1982). However, this action of TRH is abolished in hypophysectomized animals suggesting that the stimulatory effect on the thyroid is a consequence of TRH-stimulated TSH release from the pituitary (Darras and Kuhn, 1982). Furthermore, TRH (10 or 100 ng/ml medium) dose dependently increased TSH secretion in *R. pipiens*

(Denver, 1988). Also, a dose of 100 ng/ml TRH results in increased TSH secretion from *Hyla regilla* and *Xenopus laevis* pituitaries *in vitro*. In comparison, there is data which suggests that TRH does not act through the pituitary to influence the amphibian thyroid; large doses (10 μg) of TRH injected into the dorsal lymph sac or directly onto the PD failed to increase the *in vivo* thyroidal uptake of ^{131}I in *Rana temporaria* (Vandesande and Aspeslaugh, 1974). Thyroidal stimulation was not observed following the injection of 0.5 to 1.0 mg/100 g animal weight TRH into the mexican axolotl (*Ambystoma mexicanum*) (Taurog *et al.*, 1974). By comparison, TRH stimulated TSH release from the turtle (*Chrysemys picta*) pituitary at concentrations as low as 2.8×10^{-11} M (0.01 ng/ml) (Preece and Licht, 1987) suggesting that the turtle pituitary is very sensitive to TRH stimulation.

TRH also stimulated the synthesis and release of PRL from the bullfrog (*Rana catesbeiana*) adenohypophyses over a wide range of concentrations (10 nM to 10 μM) (Clemons *et al.*, 1979). Similarily, 2.8×10^{-11} to 2.8×10^{-8} M TRH stimulated PRL release from superfused turtle pituitary halves (Preece and Licht, 1987). *Rana perezi* pituitary somatotropes responded both *in vitro* and *in vivo* to TRH stimulation (Gracia-Navarro *et al.*, 1991). *In vitro*, TRH stimulated the short term release of GH while *in vivo* data suggested that TRH promotes long term synthesis (Gracia-Navarro *et al.*, 1991). Furthermore, TRH has been shown to stimulate GH release in mammals, birds and reptiles (for review, see Harvey, 1990).

TRH (10^{-9} to 10^{-6} M) stimulated α-MSH release from the amphibian (*Rana ridibunda*) NIL with an estimated ED_{50} of 1.2×10^{-8} M but α-MSH release was unaffected by several small neuropeptides such as vasoactive intestinal peptide (VIP),

morphine, ß-endorphin as well as SRIF and met-enkephalin (10^{-10} to 10^{-6} M) (Tonon *et al.*, 1983b), which modulate mammalian prolactin secretion. In comparison, neuropeptide Y (NPY) inhibited both spontaneous α-MSH release as well as TRH stimulated release from intact frog NIL's (Danger *et al.*, 1990). Dopamine (3.16×10^{-8} to 10^{-6} M) caused dose dependent reductions in α-MSH release, which were reversed by 10^{-7} M TRH (Adjeroud *et al.*, 1986). The inhibitory effects of dopamine, likely acting via a DA:D2 receptor signalling mechanism, contrast the stimulatory effects of TRH on α-MSH release from superfused frog NIL fragments suggesting that the stimulation or inhibition were independent of one another. By comparison, dopamine (10^{-9} to 10^{-5} M) induced dose-dependent decreases in α-MSH release from superfused crested newt (*Triturus cristatus*) neurointermediate lobes; however, neither TRH (10^{-7} M) nor NPY (10^{-6} M) had an effect (Danger *et al.*, 1989). By comparison, TRH (10^{-8} to 10^{-6} M) had no effect on α-MSH release from the lizard (*Lacerta vivipara*) pituitary (Dauphin-Villemant *et al.*, 1992).

Chapter 1

Thyrotropin releasing hormone (TRH) receptors in the pituitary of rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*).

ABSTRACT

Binding parameters of radiolabeled pGlu-3-Me-His^2-Pro-NH_2 ([^{3}H]MeTRH) to pituitary TRH receptors of rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*) were investigated. Washed pituitary membranes were incubated with [^{3}H]MeTRH (TRH-agonist) in the absence (B_0), or presence of TRH or TRH analogs under various paradigms. Specific binding (B_{sp}) was thermolabile and tissue dependent. Association of B_{sp} was slow (K_{+1}= 1.43 X 10^7 M^{-1} min^{-1}) achieving equilibrium binding after 60 minutes, and remaining stable for at least 60 minutes. Thereafter, [^{3}H]MeTRH binding was dissociated by addition of excess MeTRH (10^{-5} M); estimated rates of dissociation (K_{-1}) and half life ($t_{½}$) were 8.29 X 10^{-2} min^{-1} and 8.36 minutes, respectively with a kinetically derived dissociation rate constant (K_{-1}/K_{+1}) of 5.80 X 10^{-9} M. [^{3}H]MeTRH binding was displaceable; LIGAND-analysis of multiple homologous displacement experiments indicate the presence of a single class of receptors with K_d and B_{max} (±SEM) estimated as 6.93 (±2.40) X 10^{-9} M and 530 (±195) X 10^{-15} mol/mg respectively. By comparison, pGlu-His-Pro-NH_2 (native TRH) displaced [^{3}H]MeTRH from trout whole pituitary membranes in a biphasic dose-dependent manner; K_d's were estimated to be 4.15 (±3.24) X 10^{-12} M and 3.40 (±1.60) X 10^{-8} M with B_{max} = 147.68 (±10.34) and 458.85 (±50.47) X 10^{-15} mol/mg protein, respectively. Separate preparations of trout neurointermediate lobe (NIL) and pars distalis (PD) contained lower- and higher-affinity receptors, respectively, on the basis of native TRH-displacement analysis of [^{3}H]MeTRH binding. The K_d and B_{max} for the higher-affinity PD-site were 4.21 (±3.57) X 10^{-11} M and 87.41 (±12.24) X 10^{-15} mol/mg protein; for the lower-affinity NIL-site, the

K_d and B_{max} were 5.62 (±3.99) X 10^{-9} M and 252.64 (±35.37) X 10^{-15} mol/mg protein respectively. In goldfish, by comparison, the PD K_d and B_{max} were 8.77 (±6.31) X 10^{-12} M and 84.05 (±10.09) X 10^{-15} mol/mg, respectively, while the NIL-site had K_d = 2.18 (±1.56) X 10^{-9} M and B_{max} = 320.25 (±41.63) X 10^{-15} mol/mg protein. Specificity analysis of the trout pituitary TRH receptor indicates that the rank order of potency for [^{3}H]MeTRH displacement was MeTRH > TRH > pGlu-Phe-Pro-NH_2. Analogs with N- or C- terminal substitutions had little competitive potential. This study indicates the presence and binding characteristics of specific TRH receptors in the salmonid and cyprinid pituitary gland.

INTRODUCTION

Thyrotropin-releasing hormone (pGlu-His-Pro-NH_2, TRH), the first neurohormone isolated from the mammalian hypothalamus (Boler *et al.*, 1969) has also been found in the brain and pituitary gland of several tetrapod groups (Jackson, 1986) as well as in the carp (Hamano *et al.*, 1990) and sea bass (*Dicentrarchus labrax*, Teleostei) (Batten *et al.*, 1990). TRH stimulates synthesis and release of thyroid stimulating hormone (TSH) from the mammalian pituitary (Folkers *et al.*, 1970; Vale *et al.*, 1972). TRH-stimulation of TSH release has also been demonstrated in amphibians (Darras and Kuhn, 1982; Denver, 1988; Tonon *et al.*, 1983) and reptiles (Preece and Licht, 1987). In birds, TRH stimulates elevated plasma levels of thyroid and growth hormones (Leung *et al.*, 1984). In fishes, the actions of TRH are more controversial (Crim *et al.*, 1978). Some evidence suggests TRH-stimulates pituitary thyrotrophs in the dogfish via increased adenylyl cyclase activity (Deery and Jones, 1975); by comparison, Bromage (1975) suggested an inhibitory role for TRH on TSH release in the guppy (*Poecilia reticulata*) based on increased thyroid colloid density following intraperitoneal TRH-injection. Intraperitoneal TRH-injection into arctic charr (*Salvelinus aplinus*) and rainbow trout (*Salmo gairdneri*) significantly increased circulating L-thyroxine (T_4) levels which did not correlate with plasma concentrations of 3,5,3'-triiodo-L-thyronine (T_3) (Eales and Himick, 1988). Finally, Bromage *et al.* (1976) did not find a significant effect of intraperitoneally-injected TRH on circulating T_4 levels in rainbow trout.

TRH stimulates growth hormone (GH) release in mammals *in vivo* (Harvey, 1990) and from GH_1 cells in culture (Tsai and Samuels, 1974); TRH also potently stimulates

prolactin (PRL) release from GH_1 cells (Tsai and Samuels, 1974; Dannies and Markell, 1980). In amphibians TRH stimulates the release of amphibian GH (Gracia-Navarro *et al.*, 1991), PRL (Clemons *et al.*, 1979; Seki and Kikuyama, 1986) and alpha-melanocyte stimulating-hormone (α-MSH) (Tonon *et al.*, 1983). In teleosts such as the sailfin molly (*Poecilia latipinna*) TRH stimulates *in vitro* GH and PRL release depending on ambient osmotic pressure (Wigham and Batten, 1984). As well, TRH potently stimulates the release of α-MSH from goldfish NIL-fragments (Omeljaniuk *et al.*, 1989) and NIL-cells *in vitro* (Tran *et al.*, 1989) as well as adrenocorticotropic hormone (ACTH) from perifused goldfish NIL-cells (Tran *et al.*, 1989). In the rainbow trout, a TRH agonist (3-Me-His^2)TRH (MeTRH) appears to be more effective than native TRH in stimulating *in vitro* α-MSH release from perifused NIL fragments (Schwartzentruber and Omeljaniuk, chapter 2 this thesis).

Analysis of pituitary receptor binding parameters have largely used mammalian models such as the rat (Burt and Snyder, 1975; Sharif *et al.*, 1991), sheep (Sharif *et al.*, 1991; Burt and Taylor, 1980), dog (Sharif *et al.*, 1991) as well as the bovine pituitary (Sharif *et al.*, 1991; Labrie *et al.*, 1972). Specific [^{3}H]MeTRH binding sites have been demonstrated in the goldfish brain and CNS (Burt and Ajah, 1984); however, these data provide only a survey of specific TRH binding sites. Burt and Ajah (1984) provided a cursory study in the pituitaries of various marine fishes.

In this study, I have investigated the presence and binding characteristics of the pituitary TRH receptor in rainbow trout as part of an ongoing examination of TRH receptor: bioactivity relationships in the teleost brain: pituitary axis.

MATERIALS AND METHODS

Juvenile rainbow trout (*Oncorhynchus mykiss*) (25 to 150 g body weight; Rainbow Springs Hatchery, Thamesford, Ontario, Canada) were maintained in flow-through aquaria with dechlorinated water at simulated ambient temperature (5 to 16°C, annual range) and photoperiod (8 to 16h, annual range). Fish were fed Zeigler trout pellets (1 to 3% BW daily; Thunder Bay Co-op). Fish were anaesthetized with tricaine methane- sulphonate (MS-222, 0.1 g/l, Syndel, Vancouver, B.C.) and killed by spinal transection posterior to the medulla oblongata. The pituitaries were then removed and placed in ice cold (0-4°C) assay buffer (NaH_2PO_4, 20 mM; pH= 7.4). Where required, the NIL and PD were separated under a dissecting microscope by carefully teasing the individual lobes apart rather than by surgical separation using opposing scalpels thereby minimizing tissue contamination. All subsequent steps were conducted on ice.

Pituitaries were homogenized on ice in 0.1 ml of assay buffer per pituitary using 10 strokes of a motor driven Teflon-glass homogenizer (0.125 mm clearance) then transferred to 1.5 ml polypropylene microcentrifuge tubes (Fisher, Edmonton, Alta.) and centrifuged at 15 000 g X 15 minutes (4^0C). Pellets were then resuspended and diluted to appropriate concentrations and aliquots transferred to 12X75 mm polypropylene tubes.

Our receptor binding assay is based in part on Burt and Ajah, (1984). Tissue suspension (1 pituitary equivalent/ 0.1 ml) was incubated with selected concentrations of [^{3}H]MeTRH (New England Nuclear, Boston, MA; 82-87 Ci/mMol) in combination with buffer in the presence or absence of competing TRH or TRH-analogs. Incubations were terminated at various times by vacuum filtration through Whatman GF/B filters (CanLab,

Vancouver, B.C.) (presoaked overnight in assay buffer) followed by 4 rinses with 3.0 ml of ice-cold saline (0.9% NaCl). The filters were then placed in 20 ml scintillation vials (Fisher, Edmonton, Alta.), soaked overnight in ReadySafe scintillation cocktail (Beckmann, Mississauga, Ont.) then bound radioactivity was determined by liquid scintillation spectroscopy (50% counting efficiency). Protein content of tissue preparations was determined using the Bradford method (Bradford, 1976) and BSA as a standard.

Data analysis:

Kinetic data (association and dissociation data) were transformed and analyzed on the basis of Bylund and Yamamura (1990) to determine k_{+1}, k_{-1}, and K_d (see appendix A). Triplicate determinations from homologous displacement experiments allowed for LIGAND-analysis (Munson and Rodbard, 1980) to determine K_d and B_{max} (±SEM) values based upon the given parameters ± %CV (percentage coefficient of variations). Independent heterologous displacement experiments using the native TRH as a competitor, did not conform to LIGAND-analysis; therefore, data were pooled from 4 independent experiments to provide estimates of K_d and B_{max} (±SEM). Statistical comparisons are based on the 95% confidence interval where differences were considered significant at the $p<0.05$ level (Snedecor and Cochran, 1980). For clarity, the error bars (SEM's were typically less than 10% of B_{sp}) have been omitted from all figures in this chapter; however, figures with error bars have been included in appendix E.

RESULTS

1) Relationship of specifically bound [^{3}H]MeTRH to trout pituitary membrane content:

Specific binding (B_{sp}) of [^{3}H]MeTRH (1.15 to 1.30 nM) increased linearly with tissue content between 0.5 and 3.0 pituitary-equivalents/tube (approximately 15 to 80 µg protein) (Figure 1). With larger concentrations of tissue, B_{sp} increased asymptotically. In subsequent experiments, one pituitary-equivalent per tube (30 to 35 µg protein) was used generally yielding B_{sp}= 550 to 600 cpm and NSB= 200 to 250 cpm.

2) Association of [^{3}H]MeTRH to trout pituitary membrane preparation:

At 0 to 4°C, B_{sp} of [^{3}H]MeTRH (0.75 to 0.85 nM) increased slowly, reaching equilibrium (28 cpm/µg protein) after 60 minutes. Specifically bound [^{3}H]MeTRH remained relatively constant for at least 120 min. The estimated rate of association (k_{+1}) was 1.43 X 10^7 M^{-1} min^{-1} (Bylund and Yamamura, 1990) (Figure 2).

3) Dissociation of [^{3}H]MeTRH from trout pituitary membrane preparation:

Equilibrium bound [^{3}H]MeTRH (0.90 to 1.15 nM) was initially dissociated by MeTRH slowly for 40 min.; followed by a more rapid dissociation. The estimated dissociation rate (k_{-1}) was 8.29 X 10^{-2} min^{-1} (Bylund and Yamamura, 1990) accompanied by a half life ($t_{½}$) of 8.36 minutes (Figure 3). The dissociation constant (K_d) estimated on the basis of these kinetic data was 5.80 X 10^{-9} M.

4) MeTRH displacement of [^{3}H]MeTRH from whole trout pituitary membrane preparation:

MeTRH displaced [^{3}H]MeTRH (0.89 to 1.35 nM) in a dose dependent manner (Figure 4) between 10^{-11} and 10^{-6} M. LIGAND-analysis of multiple (n=3) independent experiments consistently indicated the presence of a single class of binding sites with average (mean (±SEM)) K_d= 6.93 (±2.40) X 10^{-9} M and capacity (B_{max}) of 530.6

FIG. 1. Specific binding (B_{sp}) of [^{3}H]MeTRH (expressed as cpm/μg protein) to various concentrations of washed trout pituitary membrane preparations. B_{sp} is defined as the difference between total binding (B_o), binding in the absence of competitor, and non-specific binding (NSB), binding in the presence of 10^{-5} M MeTRH. Each point represents the mean of two or three independent determinations where SEM's, typically less than 10% of B_{sp}, have not been included for clarity.

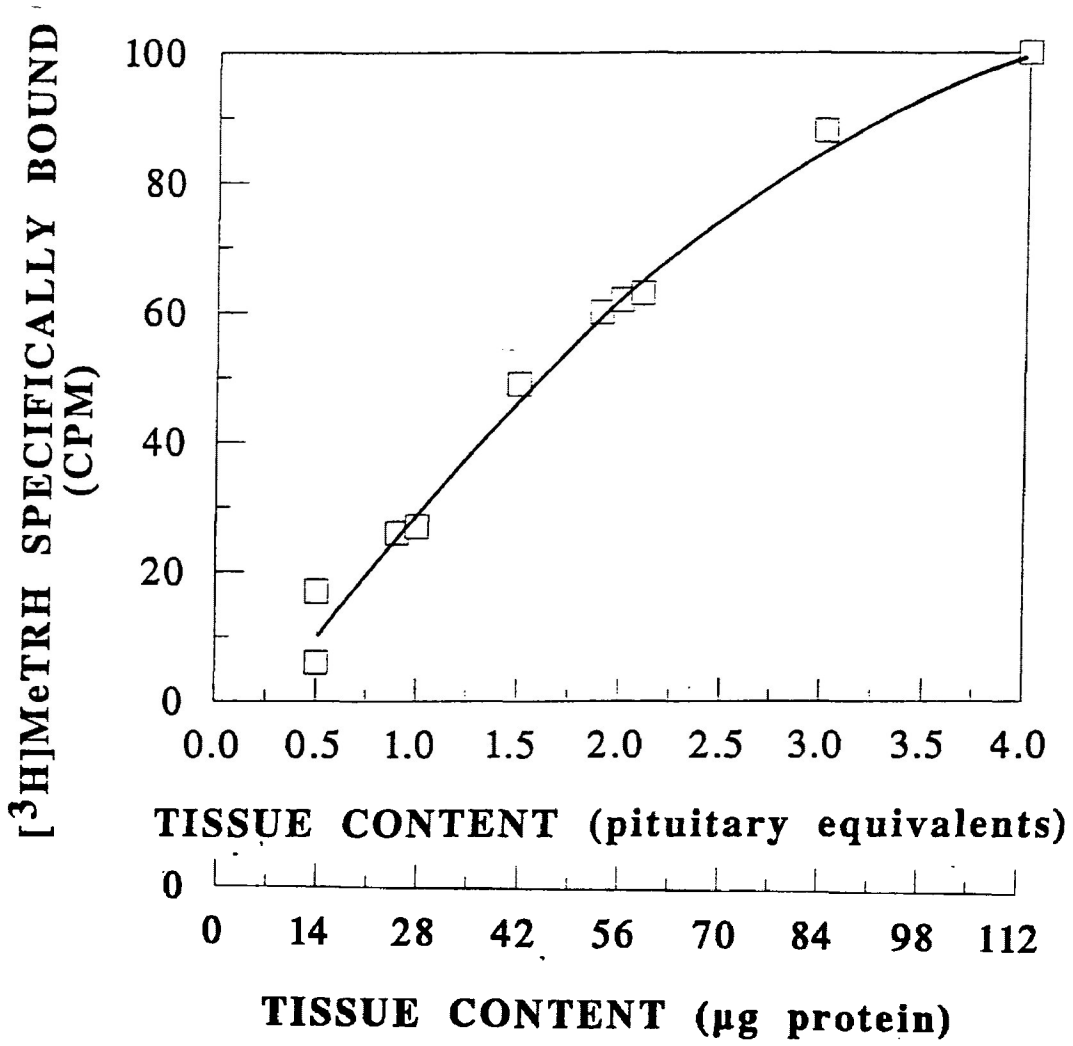

[3H]MeTRH SPECIFICALLY BOUND (CPM)
100
80
60
40
20
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0
TISSUE CONTENT (pituitary equivalents)
0
0 14 28 42 56 70 84 98 112
TISSUE CONTENT (µg protein)

FIG. 2. Association of specifically bound [^{3}H]MeTRH (cpm/μg protein) to washed trout pituitary resuspension increases as a function of time with estimated k_{+1}= 1.43 X10^{-7} M^{-1} min^{-1}. The included curve represents the line of best fit through the data where the SEM's have been omitted for clarity.

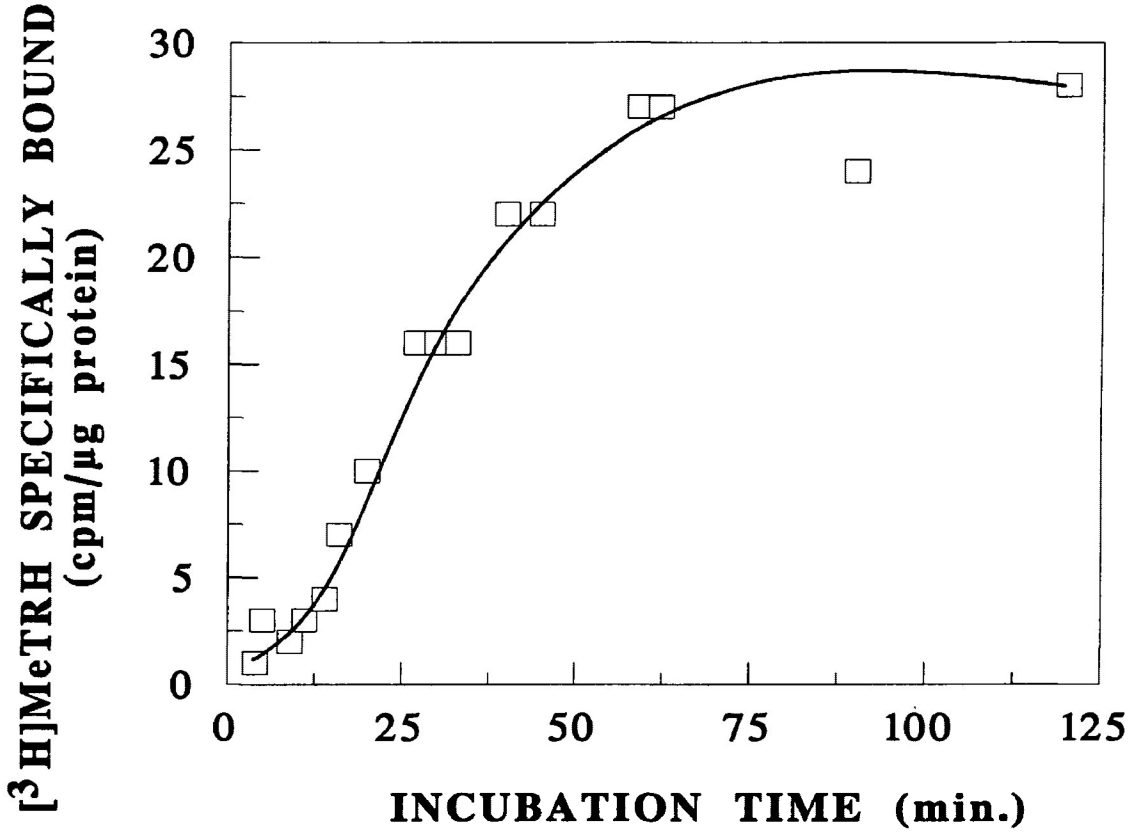

[3H]MeTRH SPECIFICALLY BOUND
(cpm/µg protein)
30
25
20
15
10
5
0
0
25
50
75
100
125
INCUBATION TIME (min.)

FIG. 3. Dissociation of maximally bound [^{3}H]MeTRH from washed trout pituitary membrane preparation following the inclusion of excess MeTRH. Dissociation was slow with a determined rate of 8.29 X 10^{-2} min^{-1}. Data are plotted with the line of best fit from 4 independent experiments (triplicate determinations) where for clarity, SEM's have been omitted.

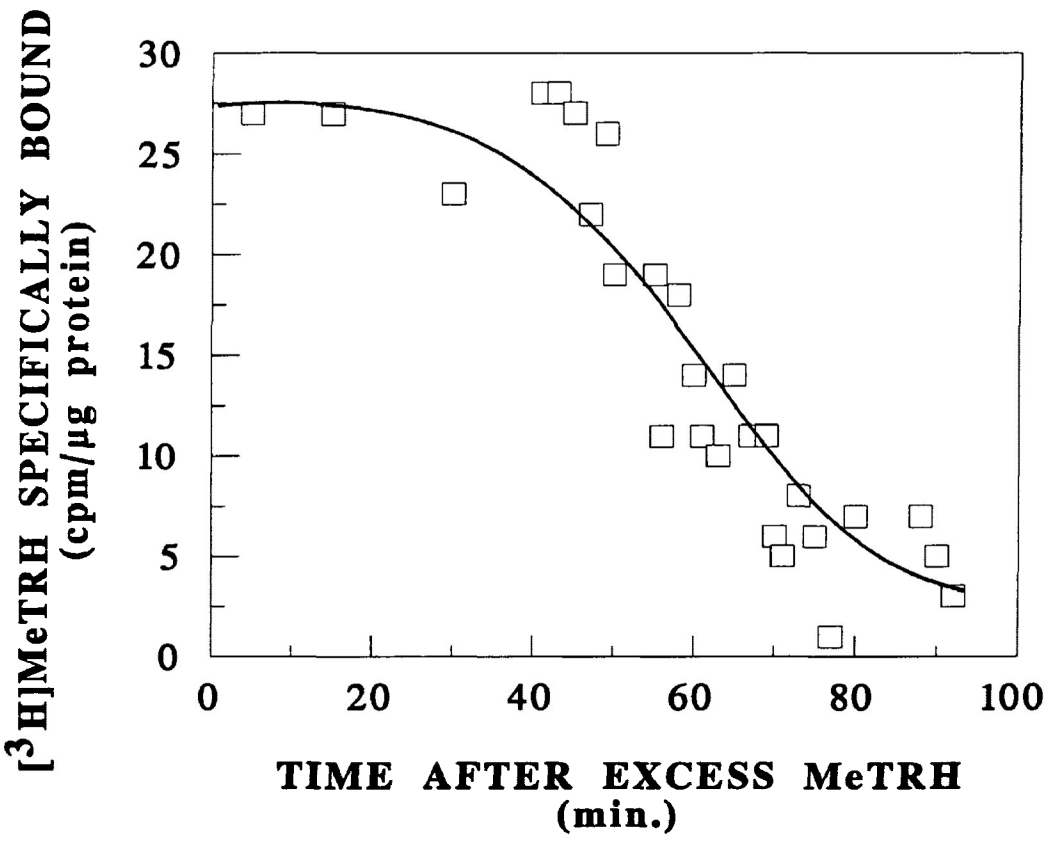
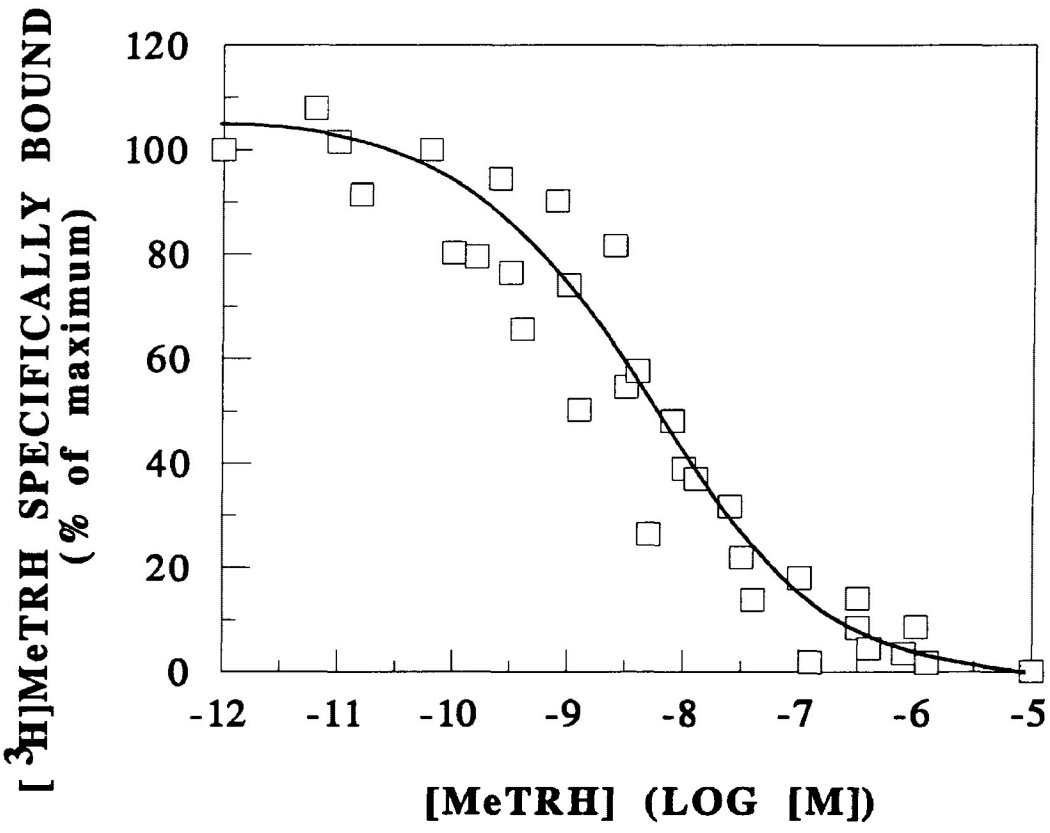

[3H]MeTRH SPECIFICALLY BOUND
(cpm/µg protein)
30
25
20
15
10
5
0
0
20
40
60
80
100
TIME AFTER EXCESS MeTRH
(min.)

FIG. 4. Homologous displacement of [^{3}H]MeTRH from trout pituitary membrane preparation. Bound radioactivity is expressed as a percentage of specific binding in the absence of competitor. MeTRH at various concentrations competed for low affinity/ high capacity receptor sites. Washed pituitary membrane preparation was incubated with a final concentration of 1 nM tracer in the presence of various concentrations of competitor. Data points represent triplicate determinations where SEM's have been omitted, pooled from 3 independent experiments.


[^{3}H]MeTRH SPECIFICALLY BOUND
(% of maximum)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
[MeTRH] (LOG [M])

(±195.1) X 10^{-15} mol/mg protein under equilibrium binding conditions.

5) TRH displacement of [^{3}H]MeTRH from trout whole pituitary membrane preparation:

pGlu-His-Pro-NH_2 (native TRH) displaced [^{3}H]MeTRH (1.05 to 1.23 nM) in a complex fashion (Figure 5). LIGAND-analysis of individual experiments did not provide estimates of K_d and B_{max}. In contrast, LIGAND-analysis of pooled data (29 triplicate determinations from 4 independent experiments; values represent the mean (±SEM)) indicates the presence of two different sites, a high affinity/ low capacity site (K_d= 4.15 (±3.24) X 10^{-12}M and B_{max}= 147.68 (±10.34) X 10^{-15} mol/mg protein) and a lower affinity/ higher capacity site (K_d= 3.40 (±1.60) X 10^{-8} M and B_{max}= 458.85 (±50.47) X 10^{-15} mol/mg protein). The binding capacities of the two sites proved to differ significantly ($P<0.05$) while the differences between K_d's were not significant.

6) TRH displacement of [^{3}H]MeTRH from trout neurointermediate lobe (NIL) and pars distalis (PD):

TRH displaced [^{3}H]MeTRH (0.92 to 1.21 nM) from both the PD and NIL in a dose- dependent manner (Figure 6). LIGAND-analysis of the data pooled from multiple PD and NIL experiments (each 27 triplicate determinations from 4 experiments) indicates the presence of specific [^{3}H]MeTRH binding sites with K_d= 4.21 (±3.57) X 10^{-11} M and B_{max}= 87.41 (±12.24) X 10^{-15} mol/mg for the PD and specific [^{3}H]MeTRH binding sites with K_d= 5.62 (±3.99) X 10^{-9} M and B_{max}= 252.64 (±35.37) X 10^{-15} mol/mg protein (values are means (±SEM)) for the NIL. Estimates for B_{max} were significantly ($P<0.05$) different between the NIL and PD; however, the corresponding K_d's were not significantly different.

7) TRH displacement of [^{3}H]MeTRH from the goldfish NIL and PD:

TRH displaced [^{3}H]MeTRH (0.88 to 1.13 nM) from the goldfish NIL and PD in much the same manner as in the trout (Figure 7). The LIGAND-estimated binding parameters (mean (±SEM)) for the PD K_d and B_{max} were 8.77 (±6.31) X 10^{-12} M and 84.05 (±10.09) X 10^{-15} mol/mg respectively when data (23 points from 3 independent experiments) was pooled. The NIL sites had an estimated K_d= 2.18 (±1.56) X 10^{-9} M and B_{max} of 320.25 (±41.63) X 10^{-15} mol/mg protein. The B_{max} estimates were significantly different ($P<0.05$) relative to the sites on the PD; however, the corresponding K_d's were not significantly different.

8) Specificity of receptor binding:

Displacement of [^{3}H]MeTRH (0.77 to 1.39 nM) from whole trout pituitary tissue preparation by TRH-analogs was structurally related (Table 1); values are means (±SEM) of triplicate determinations. At 10^{-5} M, TRH and MeTRH effectively displaced [^{3}H]MeTRH binding by 81.58 and 89.95% respectively. In contrast, [Phe^2]TRH displaced bound [^{3}H]MeTRH by 59.51%. Other analogs were variable and less potent in their ability to displace [^{3}H]MeTRH; for example, [Glu^1]TRH displaced 9% and TRH-Gly displaced 5% of bound [^{3}H]MeTRH.

DISCUSSION

These data indicate the presence of specific [^{3}H]MeTRH binding sites in the pituitary of the rainbow trout and goldfish which have binding characteristics consistent with TRH receptors in the brains and pituitaries of several species (Sharif *et al.*, 1991). TRH is a potent stimulator of α-MSH release from the goldfish (Omeljaniuk *et al.*,

FIG. 5. Displacement of [^{3}H]MeTRH with TRH from trout pituitary membrane preparation. Competition of native TRH at various concentrations in the presence of radioligand demonstrates the presence of two receptor types. Data are from triplicate determinations from independent experiments (n=4) showing an additional high affinity/ low capacity site is evident along with the same lower affinity/ higher capacity site as in Figure 4. Again, the SEM's have been omitted for clarity.

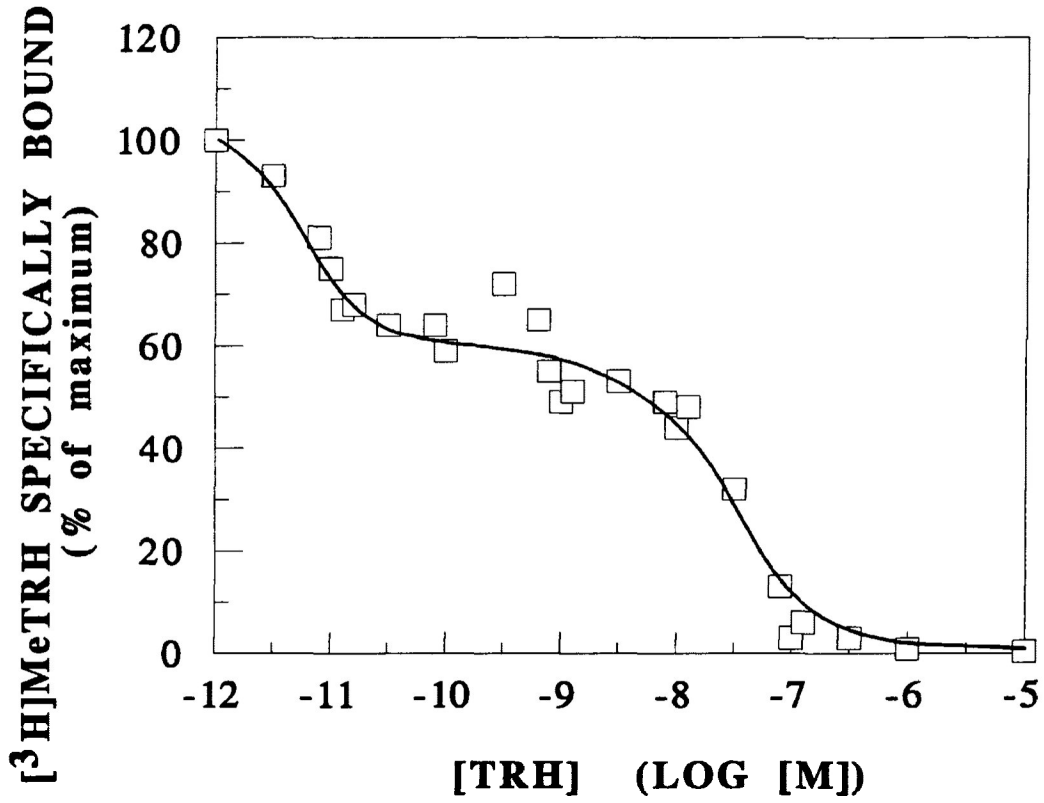

[3H]MeTRH SPECIFICALLY BOUND
(% of maximum)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
[TRH] (LOG [M])

FIG. 6. TRH displacement of [^{3}H]MeTRH from trout NIL and PD membrane preparation. Data (cpm/μg protein; 27 triplicate determinations from 4 independent experiments) are plotted as a function of competing TRH concentration (log [M]). LIGAND-analysis of displacement data indicated a class of binding sites on the NIL with nanomolar affinity and a class of binding sites having picomolar affinity on the PD.

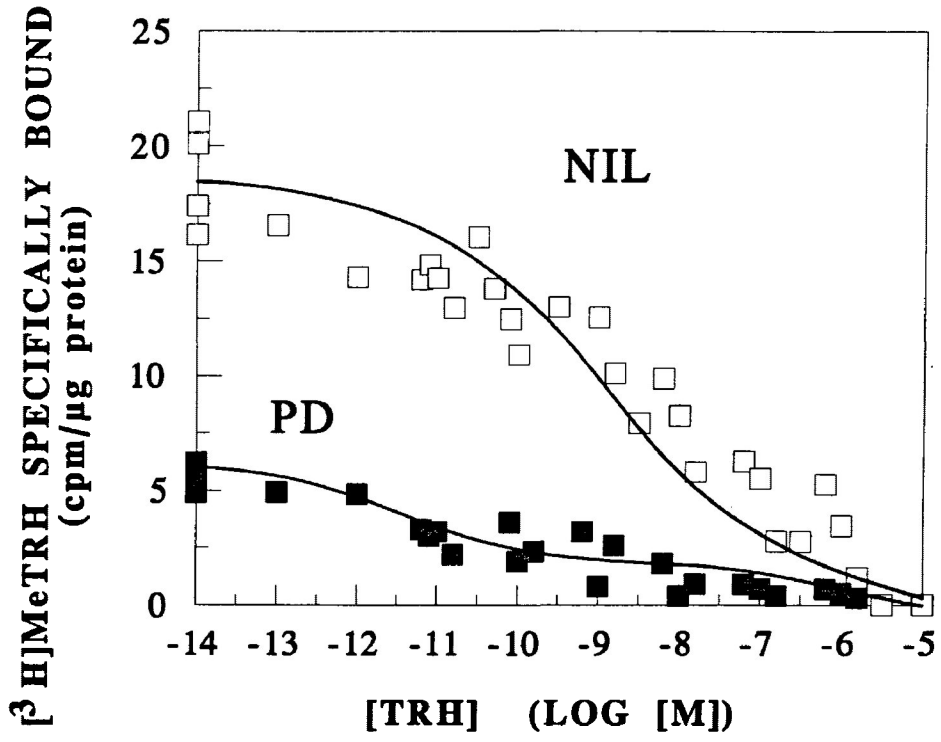

[3H]MeTRH SPECIFICALLY BOUND
(cpm/µg protein)
25
20
15
10
5
0
NIL
PD
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
[TRH] (LOG [M])

FIG. 7. TRH displacement of [^{3}H]MeTRH from goldfish NIL and PD membrane preparation. The data presented are from triplicate determinations taken from 3 independent experiments where the SEM's have been omitted for clarity. The presence of high affinity/ low capacity sites on the goldfish PD correspond to the findings in the trout (see Figure 6). Similar low affinity/ high capacity sites are found in the NIL of the goldfish as in the trout model.

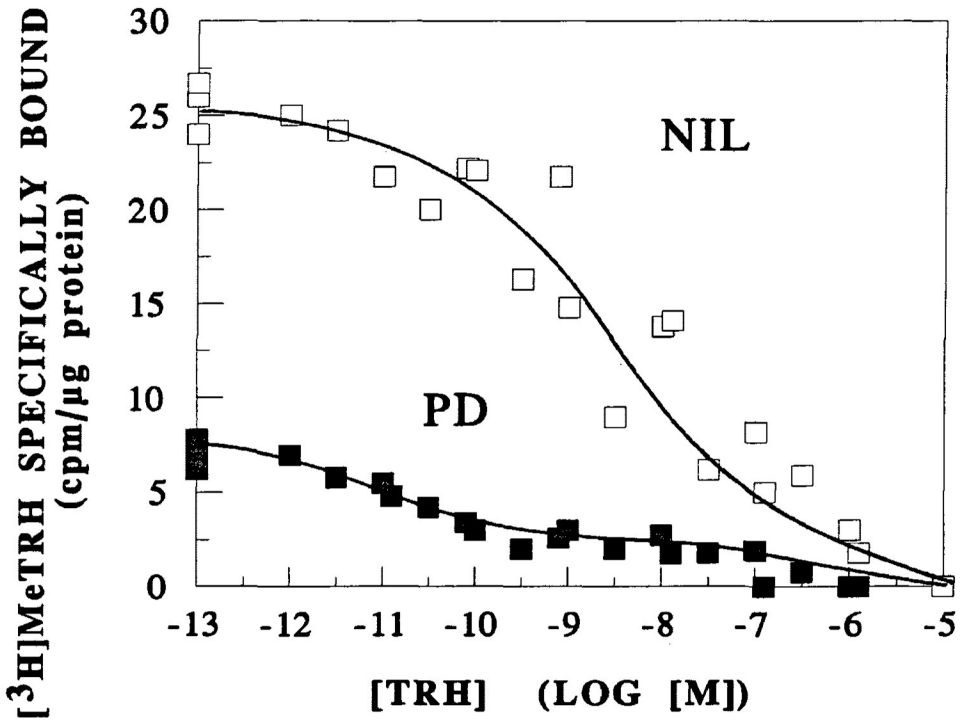

[3H]MeTRH SPECIFICALLY BOUND
(cpm/µg protein)
30
25
20
15
10
5
0
NIL
PD
-13
-12
-11
-10
-9
-8
-7
-6
-5
[TRH] (LOG [M])

TABLE 1. Displacement of [^{3}H]MeTRH from trout pituitary membrane preparation using various TRH analogs. Values are expressed as the percentage of bound radioactivity remaining in the presence of 10^{-5}M TRH-analog relative to B_o and represent the means (±SEM) of triplicate determinations from three independent experiments.

TRH Analog (10^{-5}M)	[^{3}H]MeTRH Bound (% of B_o)
pGlu-His-Pro-Gly-NH_2	95.02 ± 2.1
pGlu-His-Pro-Gly	90.36 ± 3.2
Glu-His-Pro-NH_2	82.10 ± 6.0
pGlu-His	65.66 ±10.6
pGlu-Phe-Pro-NH_2	40.49 ± 7.9
pGlu-His-Pro-NH_2 (TRH)	18.42 ± 5.8
p-Glu-3-Me-His-Pro-NH_2 (MeTRH)	10.05 ± 2.5

1989; Tran *et al.*, 1989) and trout (Schwartzentruber and Omeljaniuk, chapter 2 this thesis) NIL. As well, TRH actively stimulates the release of GH from perifused goldfish PD fragments (Trudeau *et al.*, 1992); however, I have not yet evaluated the effect of TRH on GH or PRL release from the juvenile trout pituitary. In this study, I used [^{3}H]MeTRH as a radioligand since it binds 3 to 10 times more avidly than the native ligand to mammalian pituitary TRH receptors (Hinkle, 1989); as well, [^{3}H]MeTRH has been used to identify mammalian TRH receptors in GH_4C_1 cell lines (Phillips and Hinkle, 1988) and rat, sheep, bovine, and dog anterior pituitary homogenates (Sharif *et al.*, 1991). [^{3}H]MeTRH has also been used to identify specific TRH receptors in a preliminary study of the goldfish brain and a collection of teleost pituitary tissue (Burt and Ajah, 1984).

My radioreceptor assay is based in part on an earlier preliminary examination of [^{3}H]MeTRH binding to neural and hypophyseal tissue in a mixed group of teleosts (Burt and Ajah, 1984). In my assay however, pituitaries were homogenized using a motor driven teflon-glass homogenizer and centrifuged as Burt and Ajah (1984) and the pellets resuspended using the teflon-glass homogenizer. Tissue was resuspended in a final volume of 100 µl buffer as compared to the 50 µl volume used by Burt and Ajah (1984). I believe that these differences do not preclude comparison between our data and that of Burt and Ajah (1984).

In the trout pituitary, [^{3}H]MeTRH binds reversibly in a tissue dependent manner. Specific [^{3}H]MeTRH binding is thermolabile; a single freeze-thaw cycle reduces binding by approximately 65% (data not shown). Appreciable losses in binding have been observed when frozen mammalian tissue is used under similar conditions (Burt, 1984).

LIGAND-analysis of independent homologous displacement experiments (n=3) indicates the presence of a single class of trout pituitary TRH receptors having a estimated K_d= 6.93 (±2.40) X 10^{-9}M and B_{max}= 530.6 (±195.1) X 10^{-15} mol/mg protein. Estimated association and dissociation rate constants provide a kinetically determined K_d= 5.80 X 10^{-9}M which compares with K_d's determined under equilibrium conditions. In comparison, the K_d reported here is consistent with that of mammalian pituitary TRH receptors; a range of 2.2 (±0.3) X 10^{-9}M in the rat to 9.4 (±2.5) X 10^{-9}M in the bovine pituitary (Sharif *et al.*, 1991) and the estimated K_d value for the goldfish brain of 3.74 X 10^{-9}M (Burt and Ajah, 1984). There is also a substantial range of determined maximal binding capacities within the mammalian pituitaries examined spanning approximately 20 fold (6.3 X 10^{-15} mol/mg in the dog pituitary to 128.5 X 10^{-15} mol/mg protein in the sheep pituitary) (Sharif *et al.*, 1991).

LIGAND-analysis of data pooled from multiple independent experiments using whole pituitary homogenates and TRH as a competitor reveal a class of lower-affinity receptors having K_d= 3.40 (±1.60) X 10^{-8}M as well as second class of less numerous higher-affinity receptors (K_d= 4.15 X 10^{-12}M). Separate displacement experiments did not conform to LIGAND-analysis therefore data from four independent experiments (n=29 triplicate determinations) were pooled and LIGAND-analysis could be carried out indicating the higher affinity site. Under equilibrium conditions, the higher-affinity receptors will bind a small percentage of [^{3}H]MeTRH; however, this binding is not readily displaced by the radiostable MeTRH. Conversely, the bound [^{3}H]MeTRH is displaced by the native TRH ligand thereby indicating the higher affinity sites. I propose that this

second class of pituitary TRH receptors is preferentially selective for the native ligand; however, in its absence, the receptor will bind a possible stereo-isomer of the methylated analog as was the case with our radiolabeled B_o tubes. I believe that during the production of radiolabelled MeTRH, small quantities of tracer having slightly different conformations are produced which can bind to the high affinity site while the majority of the [^{3}H]MeTRH will not recognize the site. I am presently investigating the properties of the higher-affinity site and its apparent preference for TRH.

Examination of the trout PD and NIL by heterologous displacement analysis provides evidence of a high affinity/ low capacity site on the PD having a K_d of 4.21 (±3.57) X 10^{-11}M and B_{max} of 87.41 (±12.24) X 10^{-15} mol/mg protein accompanied by a second lower affinity/ higher capacity site in the NIL with K_d= 5.62 (±3.99) X 10^{-9}M and B_{max}= 252.64 (±35.37) X 10^{-15} mol/mg. I believe that there are two distinct receptor populations differentially distributed between the PD and NIL of the trout pituitary, possibly serving two distinct physiologic functions. Although the existance of these higher affinity sites may be questionable owing to the small number of counts involved, I believe that the higher affinity sites in the PD may be associated with corticotropes which participate in the hypothalamus: pituitary: interrenal stress response pathway leading to cortisol secretion (Ball, 1981) or possibly with thyrotropes since TRH has been shown to significantly raise T_4 levels in the rainbow trout and arctic charr (Eales and Himick, 1988). In comparison, the lower affinity NIL sites are likely associated with the melanotropes as indicated by the ability to stimulate the acute dose-related release of α-MSH from the trout *in vitro* (Schwartzentruber and Omeljaniuk, chapter 2 this thesis).

The concept of distinct receptor populations for the trout NIL and PD is supported by data from the goldfish pituitary (Figure 7). LIGAND-analysis indicates a large population (320.25 (±41.63) X 10^{-15} mol/mg protein) of lower affinity (K_d= 2.18 (±1.56) X 10^{-9}M) receptors on the NIL while a second class of higher affinity/ lower capacity (K_d= 8.77 (±6.31) X 10^{-12}M and B_{max}= 84.05 (±10.09) X 10^{-15}M) exists on the PD. With respect to B_{max}, the goldfish NIL and PD receptor populations are statistically different ($P<0.05$) from each other; however, they compare with their counterparts in the trout pituitary. We believe that the lower affinity sites are responsible for the demonstrated *in vitro* stimulation of α-MSH release from NIL-fragments in the goldfish (Omeljaniuk *et al.*, 1989) and trout (Schwartzentruber and Omeljaniuk, chapter 2 this thesis) as the minimum effective dose (10^{-9}M) and ED_{50} (6.9 X 10^{-9}M) correspond to the estimated K_d of 2.18 X 10^{-9}M for the NIL receptor.

Preliminary investigations provide evidence supporting receptor specificity since substitution or interference with either the pGlu or Pro-NH_2 termini results in extremely low competition at concentrations as high as 10^{-5}M. The addition of Gly or Gly-NH_2 to the amino terminus of TRH reduces the displacement of [^{3}H]MeTRH from whole pituitary membrane preparation. Ligand-affinity is also diminished with the substitution of pGlu with the non-cyclic Glu; in comparison, substitution of the His moiety with Phe still allowed for some displacement from the lower-affinity site (Table 1). An earlier study (Sharif *et al.*, 1991) found that the addition of alkyl groups onto the proline ring or substitution of the pGlu residue with the six membered oratyl ring decreased the affinity for both CNS and pituitary receptors when compared with TRH or MeTRH in several

mammals. Analogs of TRH also exhibit strict structural specificity when competing for binding sites or prolactin producing cell lines in culture (Hinkle, 1989). *In vitro* superfusion of frog (*Rana ridibunda*) NIL fragments using 20 TRH analogs strongly suggests structural specificity since in many cases α-MSH releasing activity was reduced (Leroux *et al.*, 1982). Interestingly, MK-771 was found to increase α-MSH release in the frog (Leroux *et al.*, 1982) yet displayed decreased affinity when competing with [^{3}H]MeTRH for binding sites in the mammalian pituitary (Sharif *et al.*, 1991).

In conclusion, this study demonstrates the existence and binding characteristics of TRH receptors on the trout and goldfish pituitary. Two distinct receptor populations are segregated between the PD and the NIL in both teleosts. The more numerous NIL sites are likely associated with the melanotropes while the PD receptors may be associated with corticotropes functioning in the hypothalamus: pituitary: interrenal stress response. The biological significance of two receptor populations having differing affinities is not yet clear. Bioactivity studies, using various TRH analogs to examine receptor specificity, are described in chapter 2 of this thesis; as well, R. Omeljaniuk and I are continuing to examine the structural and molecular basis of TRH:receptor interactions.

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Chapter 2

Structural requirements for TRH stimulation of α-MSH release from rainbow trout (*Oncorhynchus mykiss*) pituitary fragments *in vitro*.

ABSTRACT

Juvenile rainbow trout pituitary glands were isolated and separated into pars distali (PD) and neurointermediate lobes (NIL). Individual lobes were then fragmented, pooled and superfused *in vitro* with acute doses of thyrotropin releasing hormone (TRH) or TRH analog; 10 minute fractions were collected and stored for subsequent analysis by specific RIA. After 2 hours of superfusion, α-MSH release from the NIL remained relatively constant; α-MSH-like immunoreactivity was not detected in PD eluate. Native TRH stimulated acute release of α-MSH from the NIL with a minimum effective dose of 10^{-9} M and estimated ED_{50} of 1.73 X 10^{-9} M on the basis of increasing dose-response experiments; decreasing dose response data provide an estimated minimum effective dose and ED_{50} of 10^{-9} and 1.57 X 10^{-9} M, respectively. No up- or down-regulatory effect was observed when NIL fragments were treated with repeated large (10^{-6} M) doses of TRH. By comparison, increasing pulse concentrations of pGlu-3-Me-His-Pro-NH_2 (MeTRH) stimulated α-MSH release with a minimum effective dose of 10^{-10} M and estimated ED_{50} of 1.56 X 10^{-9} M. Substitution of the histidine residue with phenylalanine, decreased the stimulatory actions of TRH such that the minimum effective dose was 10^{-6} M. Substitution at either the amino terminus; ([Glu^1]TRH and [1-Me-(S)-dihydroorotyl^1]TRH) or carboxy terminus (pGlu-His and TRH-Gly) resulted in near complete loss of bioactivity. This study demonstrates the strict structural requirements of the trout pituitary TRH receptor with respect to the stimulation of α-MSH release from the NIL and provides further evidence of direct TRH stimulation of melanotropes.

INTRODUCTION

Thyrotropin releasing hormone (pGlu-His-Pro-NH_2; TRH) is a phylogenetically conserved neurohormone with multiple functions. TRH stimulates synthesis and release of thyroid stimulating hormone (TSH) from the pituitary of mammals (Folkers *et al.*, 1970; Vale *et al.*, 1972), amphibians (Darras and Kuhn, 1982; Denver, 1988; Tonon *et al.*, 1983) and reptiles (Preece and Licht, 1987). In teleosts, by comparison, the actions of TRH on TSH release are not yet clear. Some evidence suggests an inhibitory role for TRH on TSH release as intraperitoneal (i.p.) injection of TRH increased thyroid colloid density indicating depressed thyroid activity in the guppy (*Poecilia reticulata*) (Bromage, 1975); conversely, i.p. injection of TRH into the rainbow trout (*Oncorhynchus mykiss*) and arctic charr (*Salvelinus alpinus*) significantly increased circulating thyroxine (T_4) levels (Eales and Himick, 1988) suggesting TRH stimulates pituitary TSH release leading to thyroid activation.

TRH is a potent stimulator of growth hormone (GH) in mammals (for review see Harvey, 1990), amphibians (Gracia-Navarro *et al.*, 1991) and fish (Wigham and Batten, 1984). As well, prolactin release is stimulated by TRH in cultured GH_1 cells (Tsai and Samuels, 1974), amphibians (Clemons *et al.*, 1979) and fish (*Poecilia latipinna*) (Wigham and Batten, 1984). In teleosts, TRH stimulates release of proopiomelanocortin (POMC) derived peptide hormones (Omeljaniuk *et al.*, 1989; Tran *et al.*, 1989). To illustrate, TRH stimulated acute release of α-melanocyte stimulating hormone (α-MSH) from superfused goldfish (*Carassius auratus*) neurointermediate lobe (NIL) fragments *in vitro* (Omeljaniuk *et al.*, 1989; Tran *et al.*, 1989); also, Tran *et al.* (1989) reported TRH stimulation of

adrenocorticotropic hormone (ACTH) from superfused dispersed goldfish NIL cells. There is no information on the structural requirements for TRH stimulation of α-MSH release in teleosts.

The teleost pituitary contains two distinct populations of highly specific TRH binding sites (Schwartzentruber and Omeljaniuk, chapter 1 this thesis). The NIL in both the rainbow trout and goldfish contains a large population of receptors with nanomolar affinity while the pars distalis (PD) contains a smaller population of picomolar affinity sites (Schwartzentruber and Omeljaniuk, chapter 1 this thesis).

The present study was conducted to investigate the structural requirements for TRH stimulation of α-MSH release from the rainbow trout pituitary and compare structural criteria for biological activity and receptor recognition in the teleost neurointermediate lobe.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS:

Fingerling rainbow trout (*Oncorhynchus mykiss*) (Rainbow Springs Hatchery, Thamesford, Ontario, Canada) were raised at the Lakehead University Aquatic Animal Research Facility in flow-through aquaria with dechlorinated water at simulated ambient temperature (5 to 16°C, annual range) and photoperiod (8 to 16h, annual range); fish were fed commercial trout pellets daily (1 to 3% body weight; Zeigler trout pellets, Thunder Bay Co-Op). Fish (75 to 100g bodyweight) were anaesthetized with tricaine methane-sulphonate (MS-222, 0.5 g/l, Syndel Laboratories, Vancouver, B.C.) prior to any handling and killed by spinal transection posterior to the medulla oblongata.

TISSUE PREPARATION:

Pituitaries were isolated in groups of seven and placed into ice- cold sterile buffer (HEPES-buffered Hank's salt solution (HBHSS); 20 mM HEPES; 0.2% bovine serum albumin, pH 7.4 at 12^{0}C). Hank's salts include: NaCl, 140 mM; KCl, 5 mM; $CaCl_2$, 1 mM; $MgCl{\cdot}6H_2O$, 0.5 mM; $MgSO_4{\cdot}7H_2O$, 0.4 mM; $Na_2HPO_4{\cdot}12H_2O$, 0.4 mM; KH_2PO_4, 0.04 mM; $NaHCO_3$, 0.4 mM; glucose, 0.6 mM; phenol red, 0.05 mM (Fisher, Edmonton, Alta.). While observing under a dissecting microscope, the PD and NIL were teased apart using scalpels as probes to minimize cross contamination of the NIL and PD tissue pools. NIL's and PD's were segregated then individual lobes (approx. 1 mm^3 PD and 2 mm^3 NIL) were sliced into approximately equally sized fragments using opposing scalpels.

***IN VITRO* SUPERFUSION:**

Fragments, collected into 12 X 75 mm polystyrene culture tubes, were suspended in 5 ml of HBHSS and centrifuged (200g for 2 min). Rinsed fragments were then loaded into superfusion chambers in a Brandel SF-6 Superfusion System (Brandel Industries, Gaithersburg, MD, USA). Each 250 µl chamber consisted of a single Whatman GF/B filter disc supporting a layer of Sephadex G-75 gel, pre-swollen for 48 hours in HBHSS, upon which pituitary fragments were placed. An additional layer of pre-swollen Sephadex G-75 gel was placed on top of the fragments and the top of the superfusion chamber was capped with another Whatman GF/B filter disc. Each chamber contained pituitary fragments from 7 individual fish; thus individual chambers represent independent replicate trials.

HBHSS was then supplied to the bottom of the chambers via peristaltic pump (300 µl/min); eluate from the top of the individual chambers was fractionally collected into polystyrene tubes, frozen and stored at -20°C for subsequent analysis of α-MSH content by specific radioimmunoassay (RIA) (Omeljaniuk *et al.*, 1989).

Specific investigations:

A) Effect of 50 pM [^{3}H]MeTRH pulses on elution of ^{3}H-radioactivity and α-MSH release:

Isolated trout NIL fragments, in three independent superfusion chambers, were superfused with HBHSS at 12°C. After 1 hour, fragments were treated with a 3 minute pulse of 50 pM [^{3}H]MeTRH (3200 cpm). Superfusion was continued for another hour, after which, 3-minute [^{3}H]MeTRH pulses were administered at 90 minute intervals. Eluate was collected into 12 X 75 mm polystyrene tubes (10 min/fraction), frozen and stored (-20°C) for subsequent determination of α-MSH content by specific RIA. When determining α-MSH content, 0.5 ml aliquots of thawed eluate were incubated overnight with 4-ml of Readysafe liquid scintillation cocktail (Beckmann, Mississauga, Ont.) in 7-ml polypropylene scintillation vials subsequently, ^{3}H-radioactivity was counted by liquid scintillation spectroscopy (50% counting efficiency).

B) Effect of repeated 10^{-6} M TRH doses on α-MSH release from trout NIL fragments *in vitro*:

NIL fragments were superfused for 2 hours with HBHSS to stabilize α-MSH release prior to treatments; thereafter, 10^{-6} M TRH was administered as 3 minute pulses at 90 minute intervals. The superfusate was collected as 10 minute fractions and stored (-20°C) for subsequent α-MSH RIA.

C) Effect of decreasing concentrations of TRH on α-MSH release from trout NIL fragments *in vitro*:

NIL fragments were superfused for 2 hours with HBHSS to stabilize α-MSH release. Subsequently TRH, from 10^{-6} to 10^{-11} M, was administered as 3 minute pulses at 90 minute intervals. 10 minute fractions were collected and stored at -20°C for α-MSH RIA.

D) Effect of increasing concentrations of TRH on α-MSH release from trout NIL fragments *in vitro*:

NIL fragments were superfused with HBHSS for 2 hours to stabilize α-MSH release. Subsequently TRH, from 10^{-11} to 10^{-6} M, was administered as 3 minute pulses at 60 minute intervals. Superfusate was collected as 10-minute fractions and stored at -20°C for subsequent α-MSH RIA.

E) Effect of increasing concentrations of TRH analogs on α-MSH release from trout NIL fragments *in vitro*:

NIL fragments were superfused for 2 hours prior to treatment to stabilize spontaneous α-MSH release. Thereafter TRH-analogs (see Appendix B for structures) were administered as 3 minute pulses from 10^{-11} to 10^{-6} M at 60 minute intervals. 10 minute fractions were collected and stored at -20°C for substquent α-MSH RIA.

HORMONE DETERMINATION

α-MSH RIA:

α-MSH content of samples was assessed by specific RIA (Omeljaniuk *et al.*, 1989). Briefly, the incubation mixture consisted of 100 µl of [^{125}I]α-MSH (approximately

5000 cpm; Peninsula Laboratories, Belmont, CA) and 300 µl of primary antibody both diluted in RIA buffer (20 mM sodium barbital, pH 8.6; sodium azide, 0.02%; bovine serum albumin, 0.3%; mercaptoethanol, 0.2%) along with 100 µl of α-MSH standard or sample in 12 X 75 mm polystyrene tubes. Following a 48 hour incubation period at 4°C, 200 µl of goat anti-rabbit gamma-globulin (GARGG-500; Peninsula Laboratories, Belmont, CA, reconstituted in 100 ml RIA buffer) and 200 µl of normal rabbit serum (NRS-500; Peninsula Laboratories, Belmont, CA; reconstituted in 100 ml RIA buffer) were added separately, on ice, to the incubation mixture. The tubes were mixed then incubated at 4°C for an additional 48 hours followed by separation of bound from free radioactivity by centrifugation (3000 RPM X 30 minutes at 4°C). Bound ^{125}I radioactivity was quantified by gamma-spectroscopy.

Probit analysis of the bound radioactivity was used to estimate α-MSH content in the eluate samples. Probit-analysis of standard curve data indicated a detection range of 5 to 2500 pg per tube (Appendix C).

DATA ANALYSIS

50 pM [^{3}H]MeTRH injected into the superfusion system eluted from superfusion chambers following an acute time course (Figure 1A); ^{3}H-radioactivity was detected in two 10-minute fractions immediately following the fraction during which the pulse was injected into the system. The time-course of ^{3}H-radioactivity elution was consistent with large acute increases of α-MSH release from NIL-fragments exposed to acute 10^{-6} M doses of TRH (Figure 2). α-MSH release responses to TRH or TRH-analogs in this *in vitro* system were contained within three 10-minute fractions immediately following the

fraction during which the pulse was injected into the system. In dynamic hormone release paradigms such as these, it is often difficult to resolve the components of total hormone release into spontaneous- and secretogougue-stimulated hormone release. In these experiments, α-MSH release following the initial two hour equilibriation period was small and not affected by 50 nM [^{3}H]MeTRH pulses (Figure 1B). These sub-threshold α-MSH release responses to 50 nM [^{3}H]MeTRH are consistent with α-MSH release from non-stimulated NIL fragments (Omeljaniuk *et al.*, unpublished data). Total hormone release into each of 10-minute fractions collected over the course of superfusion were analysed for significant differences between 90 minute intervals by the Mann-Whitney U-test (Snedecor and Cochran, 1980). Differences were considered significant at $p < 0.05$.

The effectiveness of TRH and the various TRH-analogs on stimulating α-MSH release were assessed as follows. The α-MSH released in response to a pulse of TRH or TRH-analog was included in the α-MSH released in three 10-minute fractions following the fraction during which the test substance was injected into the superfusion system ("treated" α-MSH release). By comparison, "non-treated" α-MSH release was regarded as the total hormone content in three 10-minute fractions preceding and during which the secretogogue was injected into the superfusion system. Independent, similarily treated chambers in the same experiment, at the same time, provided replicate values for "treated" and "non-treated" α-MSH release; "non-treated" values were compared with "treated" values on the basis of the Mann-Whitney U-test where differences were considered significant at $p < 0.05$. Alternately, the effects of various doses of TRH or TRH-analog were assessed by comparing the replicate "treated" values (resulting from given doses of

TRH or TRH-analog) with replicate "treated" values for the smallest dose tested within an experiment; the smallest dose, 10^{-11} M, never increased α-MSH release.

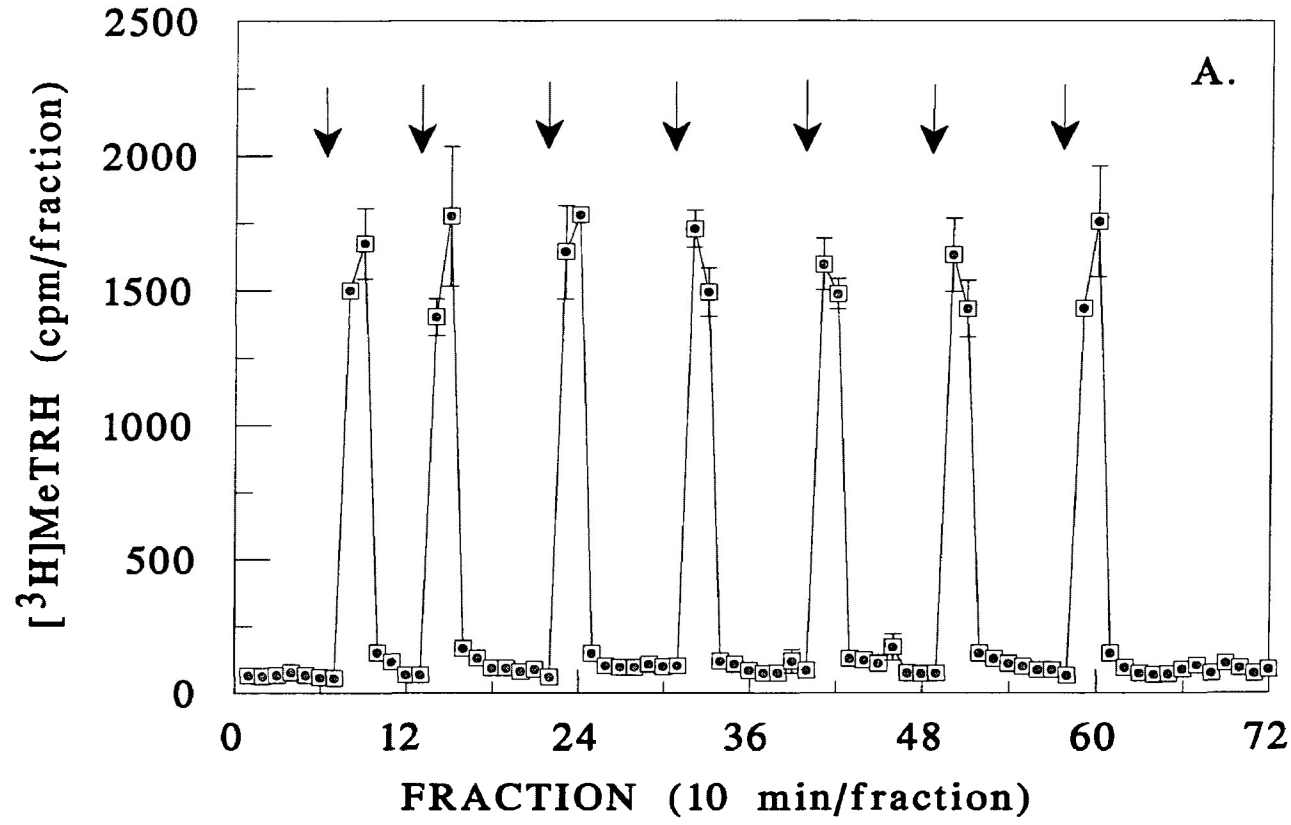
The ED_{50} (half-maximal effective dose) value was calculated on the basis of LOGIT-log transformation of dose-response data (Govindarajulu, 1988). "Treated" α-MSH release in response to 10^{-7} and 10^{-6} M TRH or MeTRH did not differ significantly ($p < 0.05$); therefore, the mean "treated" α-MSH release in response to these two concentrations was defined as the maximal response to secretogogue stimulation ("M" = 100% = 1.0) and "treated" α-MSH values (corresponding to each given dose of TRH or MeTRH) were then converted as decimal ratios of the maximal response (P). Data were transformed by ln(P/1-P). Transformed data were plotted as a function of secretogogue concentration. The half-maximal effective dose (ED_{50}) was estimated from the resulting plot as ED_{50}=-a/b; where "a" is the y-intercept and "b" is the slope of the line (Govindarajulu, 1988; see Appendix D for derivation and validation).

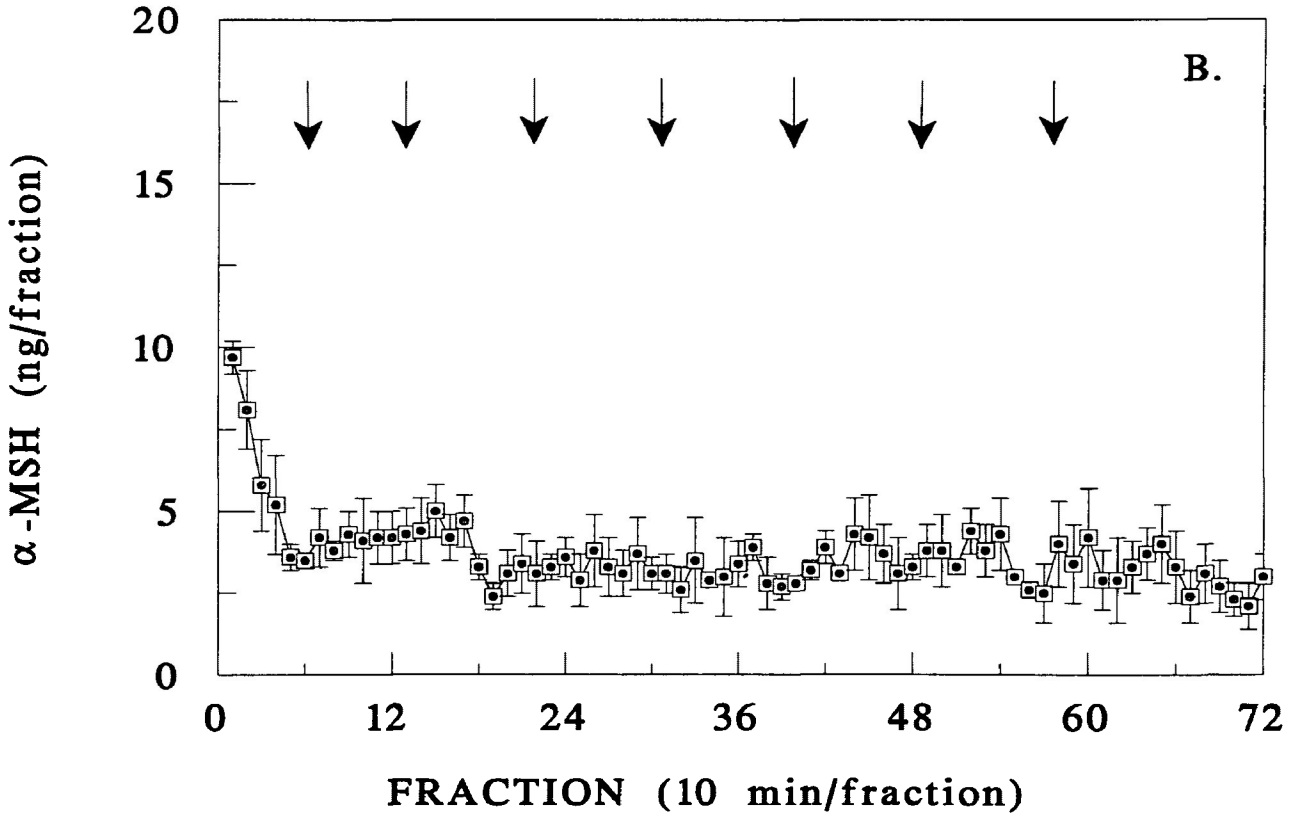
RESULTS

A) Effect of 50 pM [^{3}H]MeTRH pulses on elution of ^{3}H-radioactivity and α-MSH release:

Pulses of [^{3}H]MeTRH (3190 ± 41 cpm) eluted from superfusion chambers in an acute time-dependent manner (Figure 1A); eluted ^{3}H-radioactivity was confined to two 10-minute fractions immediately following the fraction during which the pulse was injected. Initial α-MSH release from superfused trout NIL fragments (9.7 ±0.5 ng/fraction) declined during the first 60 minutes superfusion (3.5 ±0.5 ng/fraction) to remain relatively stable for 660 minutes thereafter (Figure 1B). "Treated" α-MSH release from triplicate chambers were statistically compared and did not differ significantly ($p <$

FIG. 1. Effect of 50 pM [^{3}H]MeTRH pulses on elution of ^{3}H-radioactivity and α-MSH release. (A) ^{3}H-radioactivity (cpm/fraction) in each of 10 minute fractions collected during *in vitro* superfusion of trout neurointermediate lobe (NIL) fragments treated with 3 minute pulses of 50 pM [^{3}H]MeTRH at specific times (indicated by arrows). (B) α-MSH content of 10 minute fractions superfused with HBHSS and treated with 50 pM [^{3}H]MeTRH as 3 minute pulses at specific times (indicated by arrows). Values are plotted as the mean (±SEM) of triplicate independent determinations.


A.
2500
2000
1500
1000
500
0
[3H]MeTRH (cpm/fraction)
0
12
24
36
48
60
72
FRACTION (10 min/fraction)


B.
20
15
10
5
0
α-MSH (ng/fraction)
0
12
24
36
48
60
72
FRACTION (10 min/fraction)

0.05) over the course of the experiment (subsequent 600 min).

B) Effect of repeated 10^{-6} M TRH doses on α-MSH release from trout NIL fragments *in vitro*:

In duplicate columns, repeated pulses of 10^{-6} M TRH caused acute increases in α-MSH release (Figure 2). "Treated" α-MSH release from duplicate chambers ranged from 37.46 (±1.47) to 29.15 (±2.35) ng; by comparison, corresponding non-treated α-MSH release ranged from 8.07 (±0.19) to 2.29 (±0.66). "Treated" α-MSH release was significantly different ($p < 0.05$) compared with "non-treated" α-MSH release. The mean "treated" α-MSH release in response to six independent 3-minute 10^{-6} M TRH pulses was 34.65 (±1.14) ng; "treated" α-MSH release in response to each stimulation did not differ significantly (Table 1).

C) Effect of decreasing concentrations of TRH on α-MSH release from trout NIL fragments *in vitro*:

Decreasing concentrations of TRH-pulses caused acute dose-dependent increases in α-MSH release. In triplicate independent chambers, "treated" α-MSH release increased as a function of TRH concentration (Figure 3). The minimum effective dose required to increase "treated" α-MSH release relative to the lowest dose tested (10^{-11} M TRH did not effect α-MSH release) was 10^{-9} M. α-MSH release responses to 10^{-11} to 10^{-8} M TRH were significantly different when compared with the response to 10^{-6} M TRH; however, treated α-MSH release in response to 10^{-6} and 10^{-7} M TRH were not significantly different (Table 1). The half-maximal effective dose (ED_{50}) was estimated as 1.57×10^{-9} M (inset, Figure 3).

FIG. 2. Effect of repeated 10^{-6} M TRH doses on α-MSH release from trout NIL fragments *in vitro*. α-MSH content (ng/fraction) from 10 minute fractions collected during superfusion of NIL fragments treated with 10^{-6} M TRH (3 minute pulses indicated by arrows) at 90 minute intervals following two hours of superfusion. Values are plotted as the mean (±SEM) of duplicate experiments.

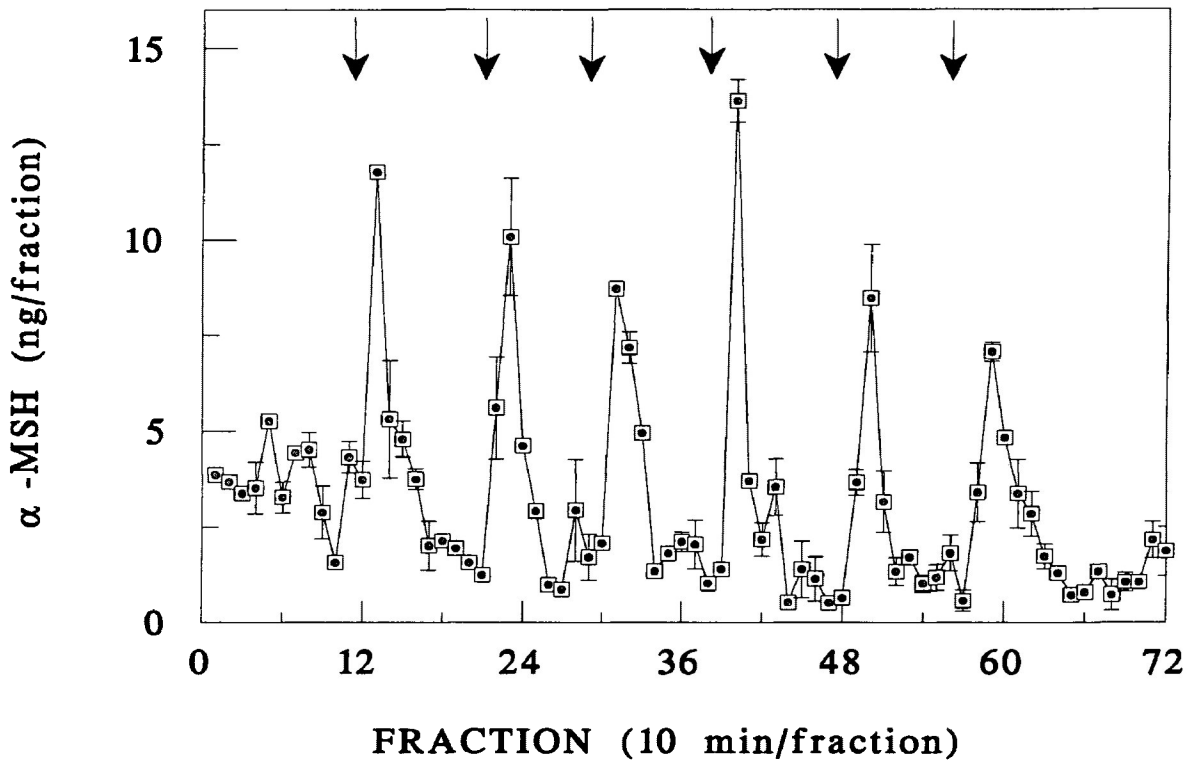
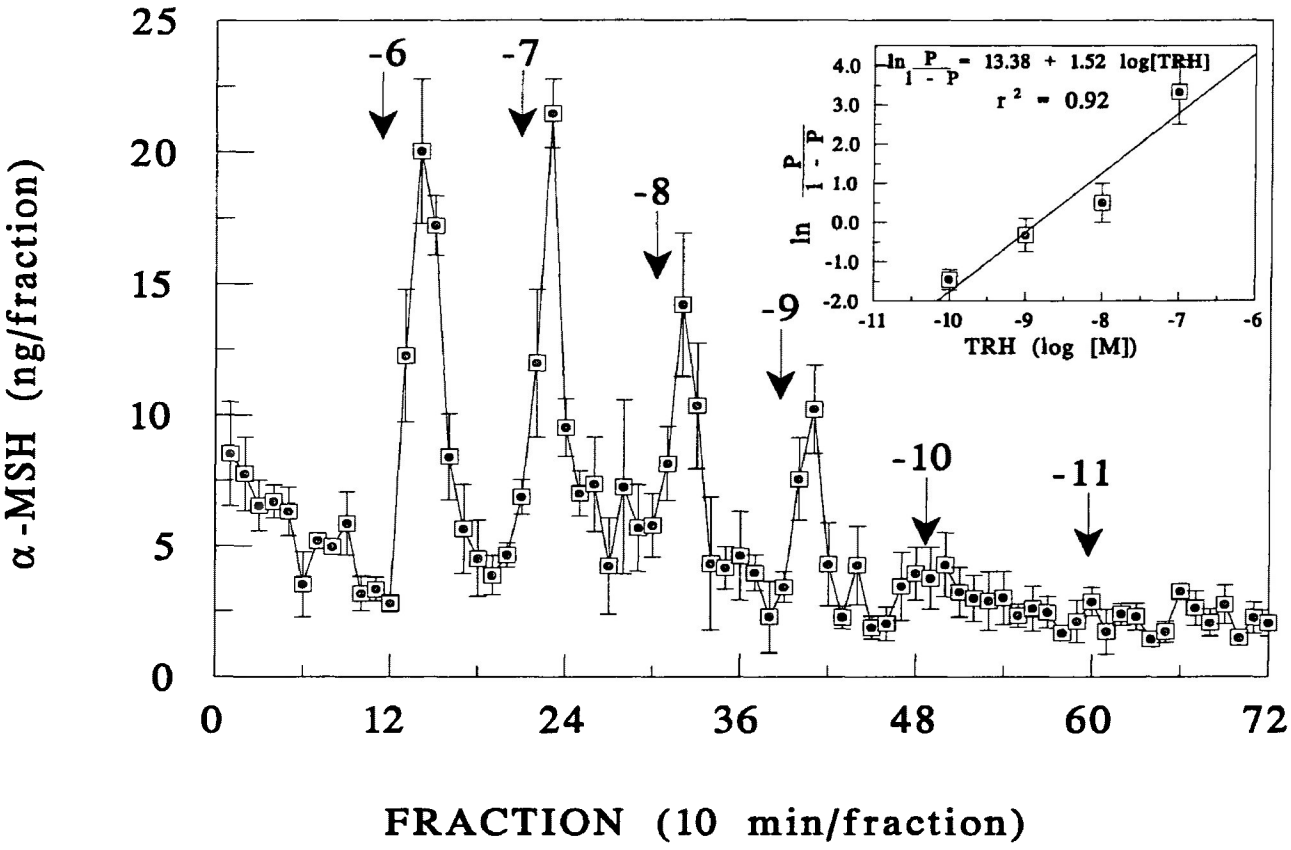

15
10
5
0
α -MSH (ng/fraction)
0
12
24
36
48
60
72
FRACTION (10 min/fraction)

FIG. 3. Effect of decreasing concentrations of TRH on α-MSH release from trout NIL fragments *in vitro*. α-MSH content (ng/fraction) from 10 minute fractions collected during superfusion with HBHSS and treated with 3 minute pulses of decreasing TRH concentrations (10^{-6} to 10^{-11} M) at 90 minute intervals. Arrows indicate the fraction during which the pulse was delivered and the concentration of secretogogue. Values are plotted as the mean (±SEM) of triplicate determinations. LOGIT-analysis (inset) of these data provided an estimated ED_{50} of 1.57 X 10^{-9} M.


α-MSH (ng/fraction)
25
20
15
10
5
0
0
12
24
36
48
60
72
FRACTION (10 min/fraction)
-6
-7
-8
-9
-10
-11
ln P/(1 - P) = 13.38 + 1.52 log[TRH]
r² = 0.92
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-11
-10
-9
-8
-7
-6
TRH (log [M])

D) Effect of increasing concentrations of TRH on α-MSH release from trout NIL fragments *in vitro*:

Increasing concentrations of TRH-pulses caused acute increases in α-MSH release. In triplicate independent chambers, "treated" α-MSH release increased as a function of TRH concentration (Figure 4). The minimum effective dose required to increase "treated" α-MSH release relative to the lowest dose tested (10^{-11} M) was 10^{-9} M. α-MSH release responses to 10^{-11} to 10^{-8} M TRH were significantly different when compared with the response to 10^{-6} M TRH; however, no significant differences were observed in α-MSH release corresponding to 10^{-6} or 10^{-7} M TRH pulses (Table 1). The half-maximal effective dose (ED_{50}) was estimated as 1.73 X 10^{-9} M (inset, Figure 4).

E) Effect of increasing concentrations of TRH-analogs on α-MSH release from trout NIL fragments *in vitro*:

Increasing concentrations of MeTRH-pulses caused acute increases in α-MSH release. In triplicate independent chambers, "treated" α-MSH release increased as a function of MeTRH concentration (Figure 5). The minimum effective dose required to increase "treated" α-MSH release relative to the lowest dose tested (10^{-11} M analog doses did not effect α-MSH release) was 10^{-10} M. α-MSH release responses to 10^{-11} to 10^{-8} M MeTRH were significantly different when compared with the response to 10^{-6} M MeTRH; however, treated α-MSH release in response to 10^{-6} or 10^{-7} M MeTRH pulses were not significantly different (Table 1). The half-maximal effective dose (ED_{50}) was estimated as 1.56 X 10^{-9} M (inset, Figure 5).

FIG. 4. Effect of increasing concentrations of TRH on α-MSH release from trout NIL fragments *in vitro*. α-MSH content (ng/fraction) from 10 minute fractions collected during superfusion with HBHSS and treated with 3 minute pulses of increasing TRH concentrations (10^{-11} to 10^{-6} M) at 60 minute intervals. Tissue was superfused for two hours prior to collection to stabilize non-stimulated α-MSH release; arrows indicate the fraction during which the TRH pulse was administered and the concentration of secretogogue. LOGIT-analysis (inset) of these data provided an estimated ED_{50} of 1.73 X 10^{-9} M.

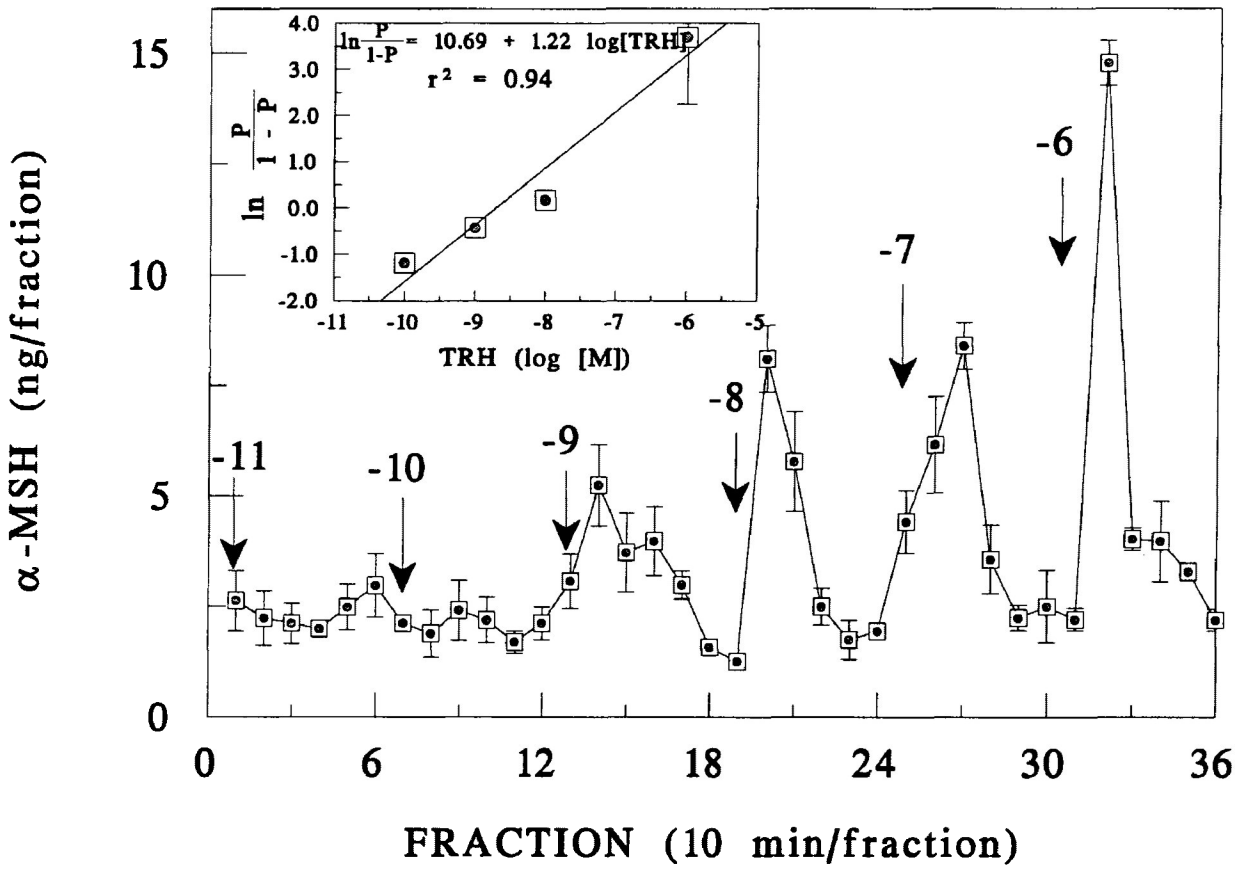
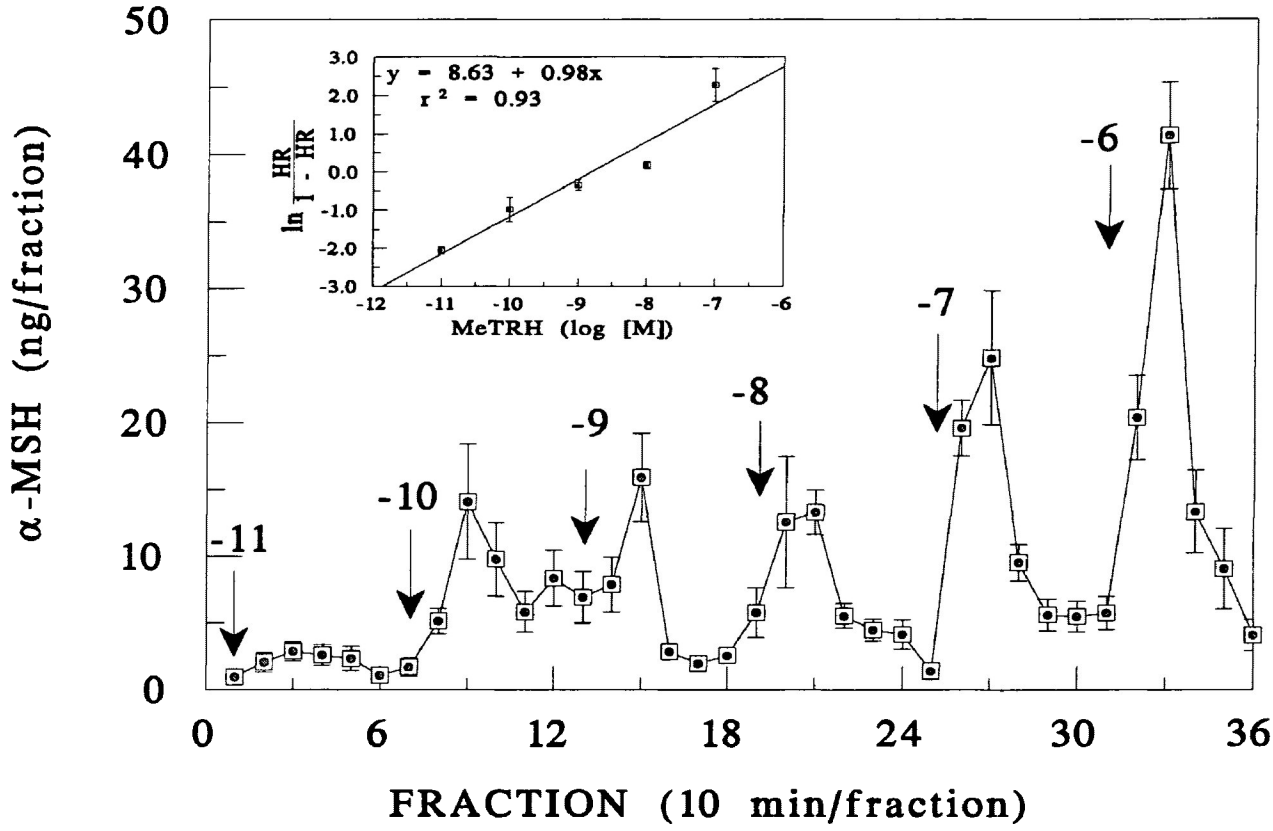

α-MSH (ng/fraction)
15
10
5
0
0
6
12
18
24
30
36
FRACTION (10 min/fraction)
-11
-10
-9
-8
-7
-6
ln P/(1-P) = 10.69 + 1.22 log[TRH]
r² = 0.94
ln P/(1-P)
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-11
-10
-9
-8
-7
-6
-5
TRH (log [M])

FIG. 5. Effect of increasing concentrations of MeTRH on α-MSH release from trout NIL fragments *in vitro*. α-MSH content (ng/fraction) from 10 minute fractions collected during superfusion with HBHSS and treated with 3 minute pulses of increasing MeTRH concentrations (10^{-11} to 10^{-6} M) at 60 minute intervals. Tissue was superfused for two hours prior to collection to stabilize non-stimulated α-MSH release; arrows indicate the fraction during which the MeTRH pulse was administered and the concentration of secretogogue. LOGIT-analysis (inset) of the dose-response data provided an estimated ED_{50} of 1.56 X 10^{-9} M.


α-MSH (ng/fraction)
FRACTION (10 min/fraction)
0
10
20
30
40
50
0
6
12
18
24
30
36
-11
-10
-9
-8
-7
-6
y = 8.63 + 0.98x
r² = 0.93
ln HR/I - HR
MeTRH (log [M])
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-12
-11
-10
-9
-8
-7
-6

TRH stimulation of α-MSH release from NIL fragments was structurally related (Table 1). Both TRH and MeTRH were effective stimulators of α-MSH release while only 10^{-6} M [Phe^2]TRH had a significant effect ($P < 0.05$) on α-MSH release. Other analogs were ineffective in affecting "treated" α-MSH release.

DISCUSSION

This project investigated the structural requirements for TRH stimulation of α-MSH release from rainbow trout neurointermediate lobe (NIL) fragments. α-MSH was released in detectable quantities from NIL fragments when superfused with HBHSS; acute exposure to 50 pM [^{3}H]MeTRH (3 minute pulses) did not effect α-MSH release from NIL fragments since this concentration was below the mimimum effective dose. α-MSH release from superfused NIL fragments decreased rapidly over the first 60 minutes of superfusion; thereafter, the α-MSH release did not vary substantially over an 11 hour period. Dopamine (DA) and apomorphine, a selective DA:D2 agonist, inhibit spontaneous α-MSH release from superfused goldfish NIL fragments in a dose-dependent manner (Omeljaniuk *et al.*, 1989) indicating dopaminergic inhibition of pituitary α-MSH release. The initially large release of α-MSH under my superfusion conditions may be attributed to the disruption of catecholaminergic inhibition of pituitary release; however, this effect is short lived and small spontaneous release remains relatively constant thereafter. This contrasts with comparatively large spontaneous gonadotropin (GtH) release from goldfish PD fragments *in vitro* (Omeljaniuk *et al.*, 1989) suggesting quantitative and qualitative differences in DA-inhibition of GtH and α-MSH release in teleosts.

TABLE 1: Effect of TRH-analogs on α-MSH release from trout NIL fragments.

	Concentration (log [M])						
	-6	-6	-6	-6	-6	-6	
TRH (repeated μM)	35.38 (±2.57)	37.38 (±2.75)	34.49 (±7.56)	37.46 (±1.47)	29.15 (±2.35)	34.03 (±2.24)	
	-11	-10	-9	-8	-7	-6	TRH -6
TRH (decreasing)	4.93 (±0.67)	6.28 (±1.23)	13.91[a] (±3.26)	20.63[a] (±4.18)	32.08[ab] (±1.38)	34.41[ab] (±5.01)	
TRH (increasing)	7.68 (±0.83)	7.74 (±1.08)	13.09[a] (±1.62)	17.98[a] (±0.64)	34.03[ab] (±0.41)	32.41[ab] (±2.37)	
MeTRH	7.42 (±0.61)	17.93[a] (±3.88)	27.24[a] (±2.26)	35.68[a] (±1.45)	59.71[ab] (±2.85)	71.99[ab] (±3.31)	
[Phe²]TRH	7.27 (±0.51)	7.99 (±2.98)	7.22 (±2.67)	14.99 (±5.43)	11.71 (±5.38)	21.87[a] (±1.66)	52.48[a] (±0.01)
[Glu¹]TRH	7.09 (±4.02)	6.11 (±1.67)	6.82 (±2.42)	6.24 (±3.50)	7.73 (±2.77)	7.04 (±4.40)	27.41[a] (±2.10)
[1-Me-5-dihydrooratyl¹]TRH	7.14 (±2.30)	7.63 (±1.29)	6.49 (±1.14)	5.32 (±0.42)	8.08 (±2.04)	6.35 (±0.84)	24.53[a] (±0.24)
pGlu-His	7.95 (±0.93)	7.46 (±0.71)	7.87 (±1.99)	7.22 (±0.89)	6.74 (±1.91)	5.94 (±1.78)	39.57[a] (±0.86)
TRH-Gly	7.58 (±2.42)	6.59 (±1.77)	5.93 (±2.74)	13.56 (±4.70)	9.36 (±5.85)	12.58 (±4.81)	47.82[a] (±1.52)

Values are means (±SEM) of triplicate independent determinations of α-MSH release from superfused NIL fragments in response to various doses of TRH or TRH-analog. "a" signifies values significantly ($p < 0.05$) different than α-MSH release in response to 10^{-11} M secretogogue; "b" represents values which were not significantly different when compared with the α-MSH release in response to 10^{-6} M secretogogue and still differed significantly when compared with the release response to the lowest concentration of secretogogue.

Typically, estimates for ED_{50} are derived by interpolation from the sigmoidal curves from plots of total stimulated hormone released versus secretogogue concentration. In this study, I have mathematically transformed (linear transformation) the dose-response data (LOGIT-analysis; Govindarajulu, 1988) and by using the y-intercept (a) and the slope (b) of the resulting line, the ED_{50} is estimated as -a/b. This method of calculation is insensitive to scale changes or choice of origin of the x-axis (Govindarajulu, 1988).

TRH stimulated acute transient dose-dependent increases in α-MSH release from trout NIL fragments. 10^{-6} M TRH pulses elicited consistant α-MSH release (34.65 ± 1.14 ng) from superfused trout NIL fragments suggesting no apparent up- or down-regulatory effect on α-MSH release. The minimum effective dose of TRH required to elicit a significant ($p < 0.05$) response was 10^{-9} M TRH while 10^{-7} M TRH had maximal effect. The estimated half maximal effective dose (ED_{50}) for TRH stimulation of α-MSH release from trout NIL fragments under these conditions was 1.73 X 10^{-9} M. Furthermore, decreasing TRH pulse concentrations stimulated dose-related increases in α-MSH release; the minimum effective dose and ED_{50} were 10^{-9} and 1.57 X 10^{-9} M respectively. Minimum effective dose and ED_{50} estimates were similar for TRH stimulation of α-MSH release as determined by both increasing- and decreasing-dose response data. By comparison, the mimimum effective dose and ED_{50} for TRH stimulation of α-MSH from superfused goldfish NIL fragments were 10^{-9} and 6.9 X 10^{-9} M respectively (Omeljaniuk *et al.*, 1989).

Of the analogs examined, only MeTRH effectively stimulated α-MSH release. The minimum effective dose was 10^{-10} M suggesting that the NIL may be more sensitive

to MeTRH than the native ligand. As with TRH, the maximum effective dose for MeTRH stimulation of α-MSH release was 10^{-7} M and the ED_{50} (1.56 X 10^{-9} M) was comparable; the estimated ED_{50} for TRH stimulation was 1.78 X 10^{-9} M. These data suggest that on the basis of ED_{50} values, there is no significant difference between the effectiveness of TRH and MeTRH with respect to the stimulation of α-MSH release. Interestingly, the total "treated" α-MSH release in response to MeTRH (71.99 ng) was substantially larger than for TRH (32.41 ng).

Substitution of the central histidine residue with phenylalanine ([Phe^2]TRH) produces an analog which does not effectively increase α-MSH release at low concentrations (minimum effective dose = 10^{-6}M). α-MSH release did not increase in response to 10^{-11} or 10^{-6} M doses of all other analogs examined suggesting strict structural requirements for TRH stimulation of α-MSH release from the trout pituitary. These data are consistent with my earlier findings concerning the structural requirements for the displacement of [^{3}H]MeTRH from rainbow trout pituitary TRH receptors where substitution at either the amino or carboxy terminus results in a near complete loss of receptor recognition (Schwartzentruber and Omeljaniuk, chapter 1 this thesis).

TRH (10^{-9} to 10^{-6} M) stimulated α-MSH release from the amphibian (*Rana ridibunda*) NIL with an estimated ED_{50} of 1.2 X 10^{-8} M; however, α-MSH release is unaffected by several small neuropeptides such as vasoactive intestinal peptide (VIP), morphine, ß-endorphin as well as SRIF and met-enkephalin (10^{-10} to 10^{-6} M), which modulate mammalian prolactin secretion (Tonon *et al.*, 1983). In comparison, neuropeptide Y inhibits both spontaneous α-MSH release as well as TRH stimulated

release from intact frog NIL's (Danger *et al.*, 1990). Interestingly, dopamine (3.16×10^{-8} to 10^{-6} M) caused dose dependent reductions in α-MSH release which were reversed in the presence of 10^{-7} M TRH (Adjeroud *et al.*, 1986). Using a similar *in vitro* perifusion system, Leroux *et al.* (1982) examined 20 TRH analogs and found that only MK-771 was equipotent when compared with TRH. The authors also found that substitution of the histidine residue at position 2 with D-His, L-Tyr, or L-Lys still allowed for stimulated α-MSH release suggesting a lesser degree of specificity required in the central position (Leroux *et al.*, 1982).

In rats, TRH stimulated the release of TSH in a dose dependent manner with a minimum effective dose of 1 ng/animal (Martin and Reichlin, 1972). In cultured GH cells, TRH stimulated TSH release with an estimated ED_{50} of 3 nM; however, MeTRH is significantly more effective stimulating TSH release with an estimated ED_{50} of less than 0.5 nM (Dannies and Markell, 1980). The short term (5 hours) stimulatory abilities of TRH and MeTRH with respect to PRL release in GH cultured cells, are not significantly different; however, after three days incubation, MeTRH stimulation of PRL release was significantly greater than the native ligand (Dannies and Markell, 1980).

In the mammalian pituitary TRH can down regulate its receptors; however, this process is slow, requiring 24 hours to reach maximum (Hinkle, 1989). Under our conditions, administration of repeated 10^{-6} M TRH pulses (Figure 2) stimulated consistent release of α-MSH above "non-treated" levels; the α-MSH release responses to TRH did not change significantly indicating that in our system TRH did not induce an up- or down-regulatory effect on α-MSH release from the trout pituitary.

In conclusion, these findings support the role of TRH as an α-MSH releasing factor in teleosts acting via highly specific pituitary TRH receptors. Under these *in vitro* conditions, MeTRH appears to be more effective than the native ligand in stimulating the release of α-MSH. Furthermore, substitution at either the N- or C- terminus produces analogs with negligible stimulatory ability.

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Chapter 3

Thyrotropin releasing hormone (TRH) receptors in the hypothalamus of rainbow trout (*Oncorhynchus mykiss*)

ABSTRACT

The existence and nature of specific [^{3}H]pGlu-3-Me-His-Pro-NH_2 ([^{3}H]MeTRH) binding sites in the rainbow trout (*Oncorhynchus mykiss*) hypothalamus were investigated. Washed hypothalamic membranes were incubated with [^{3}H]MeTRH in the absence (B_0) or presence of pGlu-His-Pro-NH_2 (TRH) or MeTRH under various experimental paradigms; incubations were terminated by filtration and bound radioactivity was determined by liquid scintillation spectroscopy. Specific binding (B_{sp}) was tissue dependent, associable, dissociable, and thermolabile. Estimated rates of association (k_{+1}) and dissociation (k_{-1}) were 1.64 X 10^7 M^{-1} min^{-1} and 1.98 X 10^{-2} min^{-1} respectively, providing a kinetically derived dissociation rate constant (K_d) of 1.21 X 10^{-9} M. [^{3}H]MeTRH binding was displaceable; LIGAND-analysis of three independent homologous displacement experiments consistently indicated a single class of binding sites with an average K_d = 6.91 (±4.32) X 10^{-9} M and estimated maximum binding capacity (B_{max}) of 8.84 (±2.72) X 10^{-15} mol/mg protein. Native TRH also displaced the radiolabel in a dose dependent manner; LIGAND-estimates for K_d and B_{max} were 1.52 (±0.12) X 10^{-9} M and 3.79 (±0.99) X 10^{-15} mol/mg protein, respectively. These data indicate the presence of a single class of specific high-affinity TRH-binding sites in the rainbow trout hypothalamus; these findings suggest a role for TRH in regulating the release of hypophysiotrophic in the teleost hypothalamus.

INTRODUCTION

The brain:pituitary axis of modern bony fishes (teleosts) provides an excellent model for studying neurohormonal regulation of pituitary function in modern bony fishes (teleosts). The teleost adenohypophysis and neurointermediate lobe are directly innervated by neurosecretory fibres; as such, neurochemicals are released directly or in proximity to target endocrine cells rather than being transported via the hypothalamo-hypophyseal blood portal system as in mammals (Peter *et al.*, 1990). In the goldfish, for example, dopamine neurons make synaptioid contacts with gonadotrophs in the pars distalis (PD) and with melanotrophs in the neurointermediate lobe (NIL) (Kah *et al.*, 1986). As well, immunoreactive thyrotropin releasing hormone (ir-TRH) nerve fibres are distributed throughout the brain and neurointermediate lobe (NIL) of the sea bass (*Dicentrarchus labrax*) pituitary (Batten *et al.*, 1990). In the NIL, ir-TRH fibres are in close proximity to groups of melanocorticotropic cells; by comparison, ir-TRH has not been detected in the pars distalis (PD) (Batten *et al.*, 1990). TRH has been shown to stimulate growth hormone (GH) (Trudeau *et al.*, 1992) and prolactin (PRL) (Wigham and Batten, 1984) release from teleost PD somatotrophs and lactotrophs respectively; as well, recent data suggest TRH activation of thyrotropes; in rainbow trout and arctic charr, intraperitoneal injection of TRH caused acute elevations in plasma L-thyroxine (T_4) (Eales and Himick, 1988).

In the carp (*Cyprinus carpio*) hypothalamus, ir-TRH neurons extend from the preoptic nucleus (NPO) to the nucleus recessus lateralis (NRL) (Hamano *et al.*, 1990). Furthermore, Hamano *et al.* (1990) indicate that the pattern of hypothalamic ir-TRH

localization resembled that of serotonin as demonstrated by Kah and Chambolle (1983) in the goldfish thereby suggesting possible interactions between TRH and serotonergic systems. In mammals, ir-TRH is widely distributed throughout the hypothalamus with large numbers of neuronal termini in both the lateral hypothalamus and periventricular nucleus (Lechan and Jackson, 1982) suggesting local neuromodulatory actions for TRH. The concept of TRH as a vertebrate neurotransmitter/neuromodulator is further supported by Poulat *et al.* (1992) who demonstrated ir-TRH synapses in the rat spinal cord.

In the rat hypothalamus, neuropeptide Y (NPY) nerve endings come in contact with TRH-producing neurons (Toni *et al.*, 1990); as well, Liao *et al.* (1991) indicate that these TRH neurons are densely surrounded by proopiomelanocortin (POMC), NPY and dopamine neurons suggesting regulation of TRH release may occur at the hypothalamic level. TRH release from perfused rat hypothalami is stimulated by dopamine and domperidone via specific DA:D2 receptors (Lewis *et al.*, 1987). Interestingly, dopamine acting at the retina inhibits TRH release in a dose-dependent manner (Mitsuma *et al.*, 1992) suggesting that dopaminergic systems may inhibit TRH release in some regions while stimulating in others. Little is known about the impact TRH has on dopamine at the hypothalamus; however, Collu *et al.* (1992) provide behavioral evidence of TRH activation of the mesolimbic dopamine system. Furthermore, DN-1417 (a TRH analog) increases tyrosine hydroxylase activity in the rat vertebral tegmental area and, at lower pH, in the striatum indicating an involvement of dopamine in the actions of TRH (Yokoo *et al.*, 1987).

TRH receptors have been characterized in the brain, spinal cord and pituitary of

a variety of mammalian species including the rat, sheep, dog, rabbit, and cow (Sharif *et al.*, 1991). Specific TRH receptors have also been characterized in the mammalian hypothalamus (Bhargava *et al.*, 1989; Funatsu *et al.*, 1985; Burt and Taylor, 1980). TRH binding sites have a wide distribution in the human hypothalamus with large numbers of receptors in the anterior and mediobasal hypothalamus while lower concentrations of TRH binding sites are evident in the posterior regions (Najimi *et al.*, 1991). I have investigated the binding parameters of [^{3}H]MeTRH to pituitary membranes of goldfish and juvenile rainbow trout and found two classes of receptors with differing affinity, capacity and tissue distribution (Schwartzentruber and Omeljaniuk, chapter 1 this thesis); to illustrate, the NIL contains a large population of receptors with nanomolar affinity while the PD contains a smaller number of receptors with dramatically higher affinity (picomolar) (Schwartzentruber and Omeljaniuk, chapter 1 this thesis). In contrast to the pituitary, I now present evidence of only a single class of specific TRH receptors in the rainbow trout hypothalamus.

METHODS

Experimental animals:

Fingerling rainbow trout (*Oncorhynchus mykiss*) (Rainbow Springs Hatchery, Thamesford, Ontario, Canada) were raised at the Lakehead University Aquatic Animal Research Facility in flow-through aquaria with dechlorinated water at simulated ambient temperature (5 to 16°C, annual range) and photoperiod (8 to 16h, annual range); fish were fed commercial trout pellets daily (1 to 3% body weight; Zeigler trout pellets, Thunder Bay Co-Op). Fish (75 to 100g bodyweight) were anaesthetized with tricaine

methanesulphonate (MS-222, 0.5 g/l, Syndel Laboratories, Vancouver, B.C.) prior to any handling and killed by spinal transection posterior to the medulla oblongata. Whole brains were removed and placed in ice cold 20 mM sodium phosphate buffer (pH = 7.4); hypothalami were carefully isolated from the remaining tissue under a dissecting microscope. The hypothalamus was defined as the region below the thalamus posterior to the telencephalon commencing at the optic tract and extending posteriorly to the nucleus diffusus lobi inferioris (landmarks from Billard and Peter, 1982; Peter and Gill, 1975). For association, dissociation and competition experiments, fresh tissue was used; however, when assessing the relationship between [^{3}H]MeTRH and tissue content, hypothalami collected earlier and flash frozen on dry ice (stored at -70°C) were used. All subsequent procedures were carried out on ice.

Radioreceptor assay:

The tissue preparation and radioreceptor assay are derived from Schwartzentruber and Omeljaniuk (submitted) with some minor changes. Briefly, hypothalami were homogenized on ice in 0.1 ml of assay buffer per hypothalamus using 10 strokes of a motor driven Teflon-glass homogenizer (0.125 mm clearance) then transferred to 1.5 ml polypropylene microcentrifuge tubes (Fisher, Edmonton, Alta.) and centrifuged at 15 000g X 15 minutes (4°C). The resulting pellets were then resuspended (hypothalamus preparation). Typically, 0.5 hypothalamus equivalents per 100 µl was incubated (4°C) with 0.75 to 1.21 nM [^{3}H]MeTRH (NEN-DuPont; Mississauga, Ontario; 84-87 Ci/mmol) in combination with buffer in the presence or absence of TRH or MeTRH in a final volume of 0.3 ml. Incubations were terminated by filtration through Whatman GF/B

filters (CanLab, Vancouver, B.C.) presoaked overnight in assay buffer containing 0.1% polyethyleneimine to reduce nonspecific binding (typically 150 to 180 cpm), followed by 4 rinses with 3.0 ml of ice-cold 0.9% NaCl. The filters were then placed in 8 ml scintillation mini-vials (Fisher Scientific, Edmonton, Alta.) and incubated overnight in 4 ml ReadySafe scintillation cocktail (Beckmann, Mississaugua, Ont.); bound radioactivity was then determined by liquid scintillation spectroscopy (50% counting efficiency).

Specific investigations:

A) Tissue dependence of [^{3}H]MeTRH binding to trout hypothalamus preparation.

Previously frozen hypothalami were homogenized in assay buffer then centrifuged; the resulting pellets were then resuspended at various concentrations and incubated with 0.75 nM [^{3}H]MeTRH in the presence (NSB) or absence (B_o) of 10 μM TRH.

B) Association of [^{3}H]MeTRH to trout hypothalamus preparation.

Hypothalamus preparation (one-half hypothalamus equivalent per tube) was incubated with [^{3}H]MeTRH (0.97 to 1.14 nM) in the absence or presence of 10 μM TRH for various intervals. Experiments were conducted in duplicate.

C) Dissociation of [^{3}H]MeTRH from trout hypothalamus preparation.

In duplicate experiments, hypothalamus preparation (one-half equivalent per tube) was incubated with [^{3}H]MeTRH (1.07 to 1.21 nM) for 70 minutes. Then TRH (10 μM final concentration) was added, tubes vortexed, and allowed to incubate for various durations.

D) MeTRH displacement of [^{3}H]MeTRH from trout hypothalamus.

In triplicate experiments, one hypothalamus equivalent per tube was incubated with

[^{3}H]MeTRH (0.87 to 0.98 nM) in the presence of various concentrations of MeTRH for 70 minutes prior to termination.

E) TRH displacement of [^{3}H]MeTRH from trout hypothalamus.

In triplicate experiments, [^{3}H]MeTRH (0.95 to 1.07 nM) was incubated with one hypothalamus equivalent per tube in the presence of various concentrations of TRH for 70 minutes prior to termination.

Protein determination:

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

Data Analysis:

Kinetic data (association and dissociation data) were transformed and analyzed on the basis of Bylund and Yamamura (1985) to determine k_{-1}, k_{+1}, and K_d. LIGAND-analysis (Munson and Rodbard, 1980) of triplicate independent experiments was used to calculate the equilibrium dissociation constant (K_d) and maximum binding capacity (B_{max}); results reported are the means (±SEM) of binding parameter determinations. Statistical comparisons of triplicate LIGAND-derived values for K_d and B_{max} were made based on the Mann-Whitney U-test; differences were considered significant at the $p<0.05$ level (Snedecor and Cochran, 1980).

RESULTS

1) Tissue dependence of [^{3}H]MeTRH binding to trout hypothalamus preparation:

Specific binding (B_{sp}) of [^{3}H]MeTRH to frozen trout hypothalamic tissue

preparation increased linearly between 0.5 and 2.0 hypothalamic equivalents/tube (approximately 225 to 850 μg protein) (Fig. 1). At larger concentrations, up to 3 hypothalamic equivalents per tube, B_{sp} increased asymptotically.

2) Association of [^{3}H]MeTRH to trout hypothalamus homogenate:

Specifically bound [^{3}H]MeTRH increased slowly for 60 minutes; thereafter, B_{sp} remained relatively constant for at least 120 minutes (Fig. 2). Data from duplicate experiments were pooled to estimate the rate of association (k_{+1}) (Bylund and Yamamura, 1990) as 1.64 X 10^7 M^{-1} min^{-1}.

3) Dissociation of [^{3}H]MeTRH from trout hypothalamus preparation:

Equilibrium bound [^{3}H]MeTRH, rapidly dissociated from the tissue preparation upon addition of 10^{-5}M MeTRH (Fig. 3). The estimated rate of dissociation (k_{-1}) (Bylund and Yamamura, 1990) based on data pooled from duplicate experiments was 1.98 X 10^{-2} M and estimated half life ($t_{½}$) was 25 minutes. The kinetically derived dissociation rate constant (k_{-1}/k_{+1}) is 1.21 X 10^{-9}M.

4) MeTRH displacement of [^{3}H]MeTRH from trout hypothalamus:

MeTRH displaced [^{3}H]MeTRH from trout hypothalamic tissue in a dose dependent manner (Fig. 4). LIGAND-analysis of three independent experiments consistently indicated a single class of binding sites with an average K_d of 6.91 (±4.32) X 10^{-9} M and estimated maximum binding capacity (B_{max}) of 8.84 (±2.72) X 10^{-15} mol/mg protein.

FIG 1: Specific binding (B_{sp}) of [^{3}H]MeTRH (cpm) to various concentrations of trout hypothalamus tissue resuspension. B_{sp} is defined as the difference between total binding (B_o), binding in the absence of competitor, and nonspecific binding (NSB), binding in the presence of 10^{-5}M TRH. Each point represents the mean (±SEM) of triplicate determinations from a single experiment.

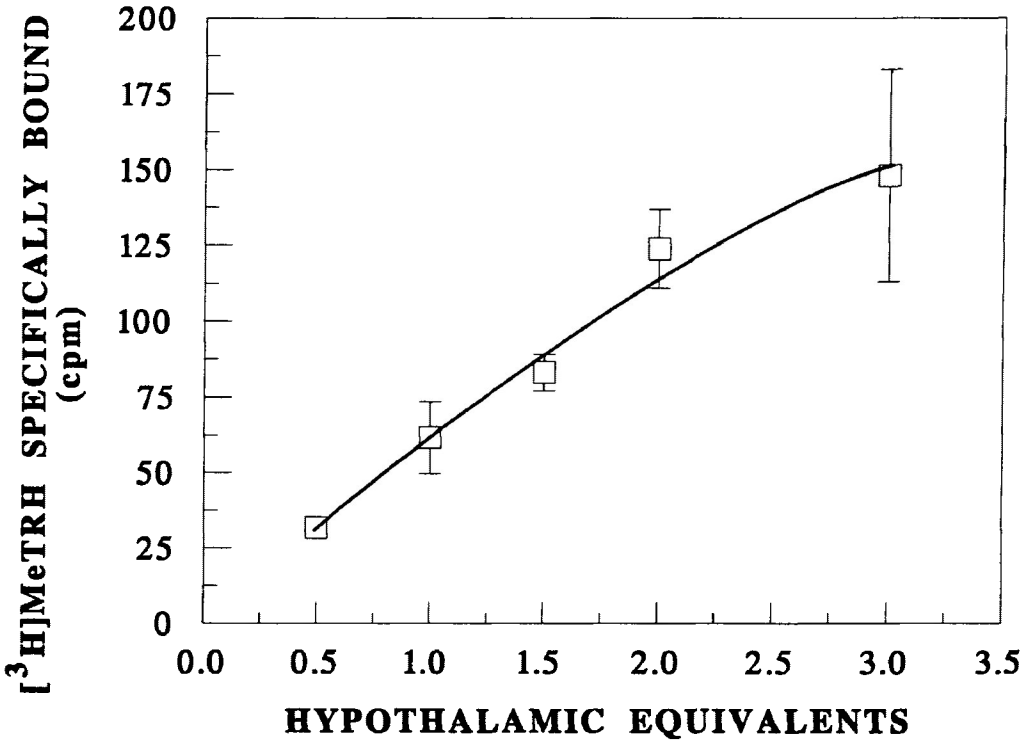

[3H]MeTRH SPECIFICALLY BOUND
(cpm)
200
175
150
125
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
HYPOTHALAMIC EQUIVALENTS

FIG 2: Association of specifically bound [^{3}H]MeTRH (cpm) to washed trout hypothalamus preparation. B_{sp} increases over time reaching near maximum after 60 minutes. The estimated rate of association (k_{+1}) is 1.64 X 10^7 M^{-1} min^{-1}. Data represent the means (±SEM) of triplicate determinations from duplicate experiments.

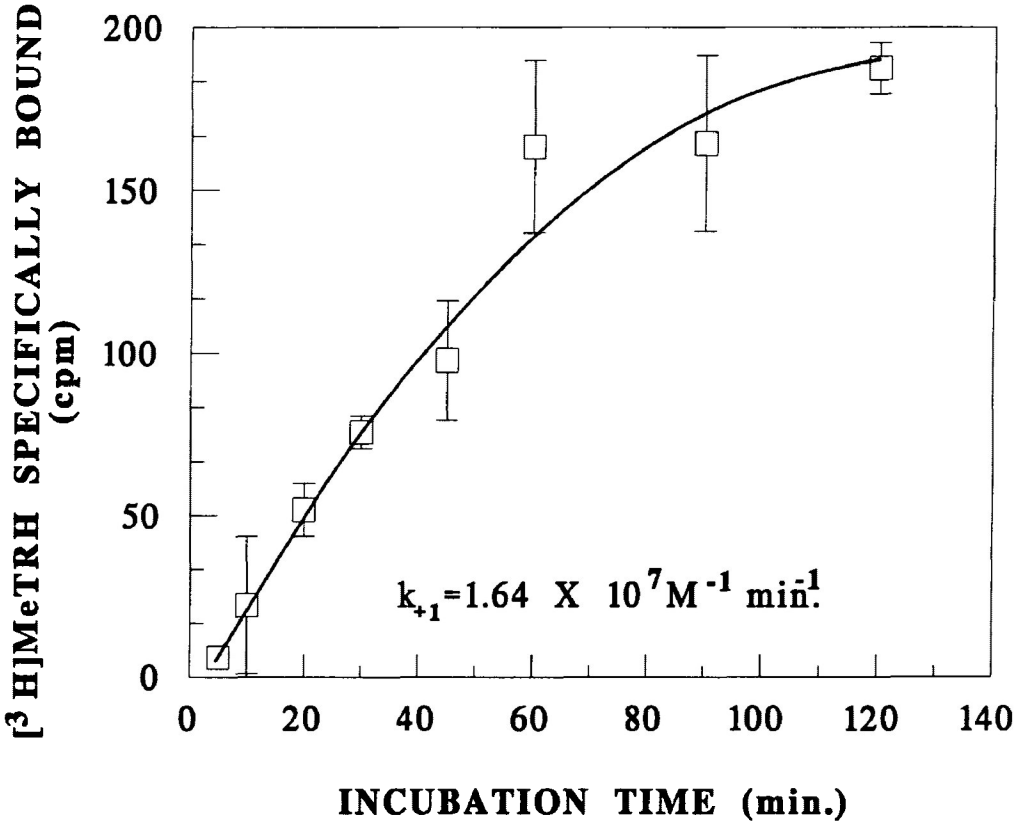

[3 H]MeTRH SPECIFICALLY BOUND (cpm)
200
150
100
50
0
0
20
40
60
80
100
120
140
INCUBATION TIME (min.)
k+1=1.64 X 10 7M -1 min-1

FIG 3: Dissociation of equilibrium bound [^{3}H]MeTRH (cpm) from washed trout hypothalamus membrane preparation following the inclusion of 10^{-5}M TRH. Dissociation was initially rapid with an estimated half-life ($t_{½}$) of 25 minutes and dissociation rate (k_{-1}) of 1.98 X 10^{-2} min^{-1}. Data are the means (±SEM) of triplicate determinations from duplicate experiments.

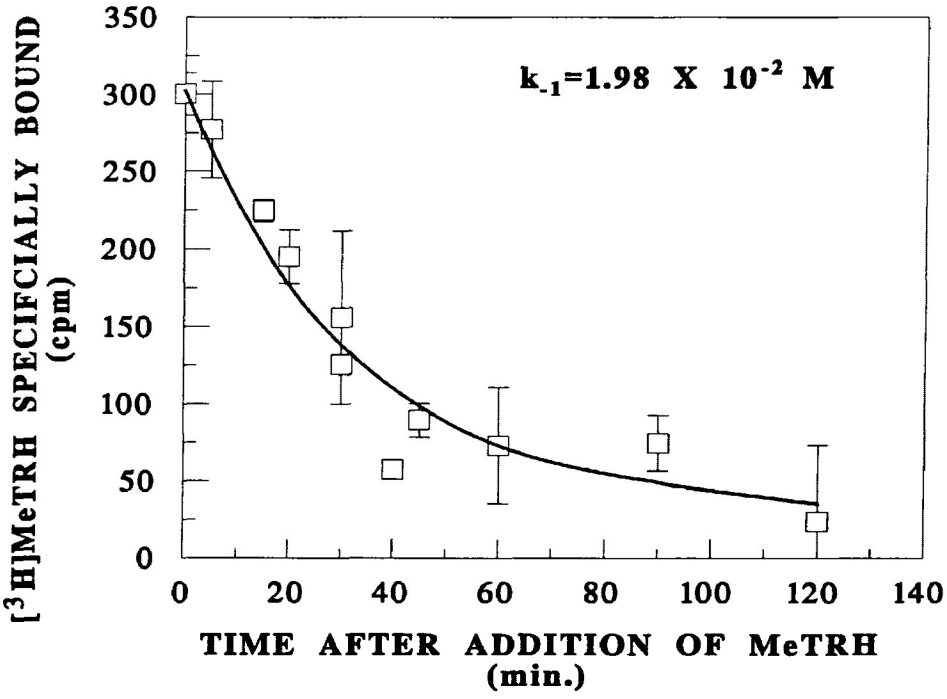
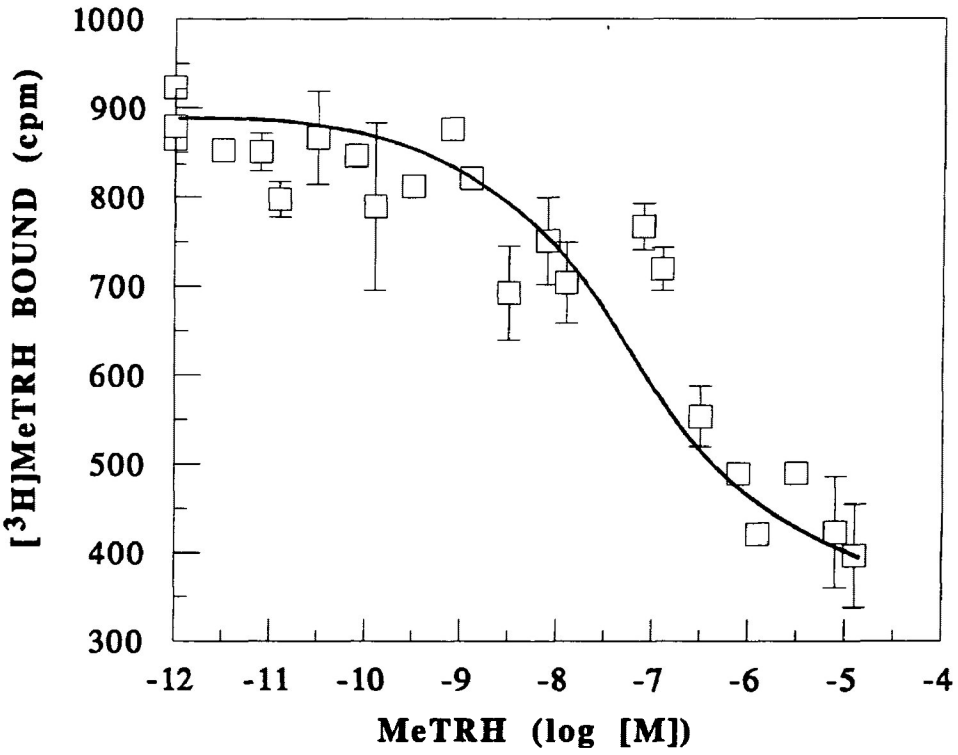

k_{-1}=1.98 X 10^{-2} M
[^{3}H]MeTRH SPECIFCIALLY BOUND (cpm)
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
140
TIME AFTER ADDITION OF MeTRH (min.)

FIG 4: [^{3}H]MeTRH bound as a function of MeTRH concentration (log [M]). LIGAND-analysis consistently indicated a single class of binding sites with an estimated $K_d = 9.61\ (\pm 4.32) \times 10^{-9}$M and $B_{max} = 8.84\ (\pm 2.72) \times 10^{-15}$ mol/mg protein (mean ±SEM; n=3 experiments). Data are plotted as the means (±SEM) of triplicate determinations from triplicate experiments.


1000
900
800
700
600
500
400
300
[3H]MeTRH BOUND (cpm)
-12
-11
-10
-9
-8
-7
-6
-5
-4
MeTRH (log [M])

5) TRH displacement of [^{3}H]MeTRH from trout hypothalamus:

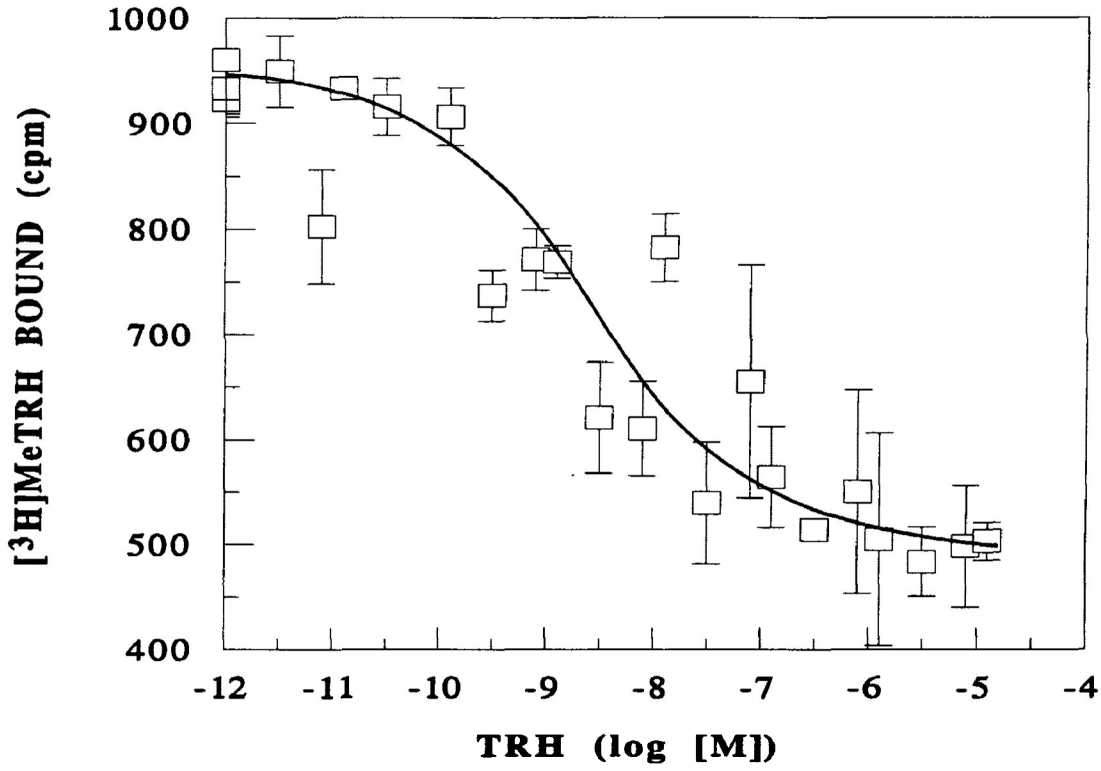
[^{3}H]MeTRH was displaced from trout hypothalamus by TRH in a dose dependent manner (Fig. 5). LIGAND-analysis of data from duplicate determinations indicated a single class of binding sites with K_d = 1.52 (±0.12) X 10^{-9} M and B_{max} = 3.79 (±0.99) X 10^{-15} mol/mg protein (values are means ±SEM; n=3 experiments).

DISCUSSION

My data indicate the presence of a single class of specific TRH binding sites in the trout hypothalamus. TRH, a potent hypothalamic releasing factor, stimulates the synthesis and release of several hypophyseal hormones in a variety of animal groups. For example, TRH actively stimulates the release of thyroid stimulating hormone (TSH) from the mammalian (Folkers *et al.*, 1970; Vale *et al.*, 1972), amphibian (Denver, 1988; Castano *et al.*, 1992), and reptilian pituitary (Preece and Licht, 1987). In teleosts, by comparison, the role of TRH is more controversial with some evidence suggesting a stimulatory role on the pituitary-thyroid axis (Eales and Himick, 1988) while other evidence suggests an inhibitory role (Bromage, 1975).

Prolactin (PRL) release, stimulated by TRH, has been well documented in mammals (Mena *et al.*, 1989; Haisenleder *et al.*, 1991), amphibians (Castano *et al.*, 1992; Clemons *et al.*, 1979; Nakajima *et al.*, 1993; Sakai *et al.*, 1991), reptiles (Preece and Licht, 1987) and teleosts (Wigham and Batten, 1984). The ability of TRH to stimulate the release of growth hormone (GH) has also been demonstrated in mammals, amphibians and birds (for review see Harvey, 1990). Recently, TRH has been shown to stimulate GH release in both sexually mature and sexually regressed goldfish

FIG 5: [^{3}H]MeTRH bound as a function of TRH concentration (log [M]). Ligand analysis of independent experiments consistently indicated a one-site fit; estimates of the binding parameters (expressed as the mean (±SEM) from triplicate experiments) are: K_d= 1.52 (±0.12) X 10^{-9}M and B_{max}= 3.79 (±0.99) X 10^{-15} mol/mg protein. Data are plotted as the means (±SEM) of triplicate determinations from three independent experiments.


1000
900
800
700
600
500
400
[3H]MeTRH BOUND (cpm)
-12
-11
-10
-9
-8
-7
-6
-5
-4
TRH (log [M])

(Trudeau *et al.*, 1992).

TRH is also a potent stimulator of proopiomelanocortin (POMC) derived peptide hormones from the teleost pituitary. Superfused goldfish (*Carassius auratus*) neurointermediate lobe (NIL) fragments respond to TRH stimulation by concomitantly releasing α-melanocyte stimulating hormone (α-MSH) (Omeljaniuk *et al.*, 1989; Tran *et al.*, 1989) and adrenocorticotropic hormone (ACTH) (Tran *et al.*, 1989). In the rainbow trout (*Oncorhynchus mykiss*), both TRH and (3-Me-His^2)TRH (MeTRH), a mammalian TRH agonist, actively stimulate α-MSH release from superfused NIL fragments (Schwartzentruber and Omeljaniuk, chapter 2 this thesis).

In the trout hypothalamus, [^{3}H]MeTRH binds reversibly in a tissue dependent manner. Specific [^{3}H]MeTRH binding is thermolabile; a single freeze-thaw cycle reduces binding by approximately 80 % (unpublished). These findings are similar to decreases in B_{sp} we have noted when trout pituitary glands are subjected to a single freeze-thaw cycle (Schwartzentruber and Omeljaniuk, chapter 1 this thesis); furthermore, appreciable losses in binding have also been observed when frozen mammalian tissue is used under similar conditions (Burt, 1984).

In the trout hypothalamus, MeTRH displacement of [^{3}H]MeTRH consistently revealed a single class of binding sites with an estimated K_d = 9.61 (±4.32) X 10^{-9} M and capacity of 8.84 (±2.72) X 10^{-15} mol/mg protein. The estimated K_d compares with the trout pituitary where LIGAND-analysis of MeTRH displacement data indicates a single class of receptors (K_d = 6.93 (±2.40) X 10^{-9} M); however, the maximum binding capacity of the hypothalamus is significantly lower ($P < 0.05$) than the pituitary (B_{max} = 530 (±195)

x 10^{-15} mol/mg protein) (Schwartzentruber and Omeljaniuk, chapter 1 this thesis). Displacement of [^{3}H]MeTRH by TRH consistently showed a single class of receptors having an estimated K_d of 1.52 (±0.12) X 10^{-9} M and B_{max} of 3.79 (±0.99) X 10^{-15} mol/mg protein; the binding parameters were not statistically different ($P < 0.05$) when compared with values obtained from homologous displacement experiments. However, TRH displacement of [^{3}H]MeTRH from the trout pituitary revealed two distinct classes of receptors based on their K_d's and capacities. These classes were determined to exist as separate populations where the neurointermediate lobe (NIL) contained a large population (B_{max} = 252.64 (±35.37) X 10^{-15} mol/mg protein) of nanomolar affinity receptors (K_d = 5.62 (±3.99) X 10^{-9} M) while the pars distalis (PD) contained a smaller number (B_{max} = 87.41 (±12.24) X 10^{-15} mol/mg protein) of higher affinity receptors (4.21 (±3.57) X 10^{-11} M) (Schwartzentruber and Omeljaniuk, chapter 1 this thesis).

The affinity of the hypothalamic receptors is similar to the affinity of NIL receptors (Schwartzentruber and Omeljaniuk, chapter 1 this thesis); however, the hypothalamus contains significantly fewer receptors ($P < 0.05$). By comparison, the hypothalamus lacks the higher affinity TRH receptors (picomolar) present in the trout pars distalis (PD) (Schwartzentruber and Omeljaniuk, chapter 1 this thesis). I have no data which would clarify the genomic origin of the TRH receptors in the trout PD, NIL, and hypothalamus. However, it is interesting to recognize that the nanomolar affinity TRH receptor appears to be restricted to tissue of neural origin (hypothalamus, NIL) while the picomolar affinity site is restricted to the endodermally derived PD. I believe that the distinct pituitary receptor types may serve different roles with the nanomolar affinity sites

regulating α-MSH release form the NIL (Omeljaniuk *et al.*, 1989; Tran *et al.*, 1989) while the picomolar affinity sites on the PD may function in the regulation of GH release (Trudeau *et al.*, 1989).

My data confirm and extend the preliminary findings of Burt and Ajah (1984) who demonstrated that the inferior lobe of the goldfish brain (which contained the hypothalamus) contained receptors with nanomolar affinity with an estimated capacity of 10.8 (±1.5) X 10^{-15} mol/mg protein. Burt and Ajah (1984) also presented evidence of an additional class of lower affinity receptors ($K_d \approx 15$ μM) distributed throughout the brain which we did not detect in the trout brain; I am not aware of a physiologic function for TRH consistent with this very low affinity site.

The mammalian hypothalamus contains highly specific TRH receptors with an estimated K_d = 1.4 (±0.3) X 10^{-9} M and capacity of 10 (±3) X 10^{-15} mol/mg protein (Funatsu *et al.*, 1985). These receptors may function in the maintenance of body temperature since TRH injection into the preoptic/anterior hypothalamus decreases the thermoregulatory set point in ground squirrels (Hendriksen *et al.*, 1992). In comparison, autoradiography of human TRH binding sites indicates a single class of binding sites with an estimated k_d = 4.8 (±0.6) X 10^{-9} M and capacity of 147 (±14) X 10^{-15} mol/mg tissue (Najimi *et al.*, 1991). These findings are similar to the trout hypothalamus in both affinity and capacity.

The presence of hypothalamic TRH receptors in the trout is consistent with TRH distribution in the teleost brain. Immunoreactive TRH (ir-TRH) has been identified in the neuronal processes of the teleost hypothalamus extending from the preoptic nucleus to the

nucleus recessus lateralis (Hamano *et al.*, 1990). In comparison, ir-TRH neurons characterized in the mammalian hypothalamus and are found predominantly in the periventricular nucleus (PVN) projecting to the median eminence (Lechan and Jackson, 1982). TRH cell bodies located in the PVN are in close association with proopiomelanocortin (POMC) neurons, neuropeptide Y (NPY) neurons, as well as dopamine-ß-hydroxylase (DßH) nerve fibres suggesting possible TRH regulation by POMC, NPY, or adrenergic/noradrenergic systems (Liao *et al.*, 1991). Recent studies from rat studies suggest that TRH may activate the mesolimbic dopamine neurons to elicit selected stereotypic behaviour (Collu *et al.*, 1992). To the best of my knowledge, there is no information on brain or hypothalamic TRH-receptor function in non-mammalian vertebrates.

Hypothalamic release of TRH is increased by dopamine (DA) and norepinephrine (NE); moreover, the stimulatory effect exhibited by DA is blocked by disulfiram, a dopamine-ß-oxidase inhibitor, suggesting the DA effect occurs after DA is converted to NE (Grimm and Reichlin, 1973). Furthermore, hypothalamic TRH release is stimulated by DA, L-dopa, and bromocryptine but this effect is blocked by haloperidol, a specific dopamine D2-receptor antagonist (Maeda and Frohman, 1980). Interestingly, intraventricular infusion of TRH stimulates DA release from rat tuberoinfundibular neurons in a dose/time dependent manner with a corresponding reduction in hypothalamic TRH binding capacity (B_{max}) but not K_d (Ikegami *et al.*, 1992). In addition, histamine and dimaprit, a histamine H2 agonist, stimulate TRH release from rat hypothalamic fragments; this stimulatory effect is blocked by metiamide (H2 antagonist) but not mepyramine (H1

antagonist) (Joseph-Bravo *et al.*, 1979). The effects of serotonin (5HT) on TRH release from the hypothalamus are controversial with evidence suggesting an inhibitory role at high concentrations (10^{-4}M) (Grimm and Reichlin, 1973) while a stimulatory effect has been shown with low concentrations (2×10^{-12} and 2×10^{-10} M) (Chen and Ramirez, 1981) furthermore, inconclusive results, 5HT at concentrations ranging from 10^{-6} to 10^{-4} M, were reported by Maeda and Frohman (1980). These findings strongly support complementary regulation of several hypophyseal releasing factors at the preoptic/hypothalamic level in mammals.

In conclusion, I present evidence of a single class of high affinity TRH receptors in the trout hypothalamus. The presence of these receptors suggests that TRH may mediate hypothalamic/preoptic neuronal activity influencing pituitary function in teleosts.

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DISCUSSION

In this study, the existence and binding parameters of specific pituitary TRH receptors in the trout and goldfish were examined; specific TRH receptors were also characterized in the trout hypothalamus. Furthermore, the structural requirements for TRH-stimulation of α-MSH release from superfused trout NIL fragments were also examined. Homologous ([^{3}H]MeTRH displaced by MeTRH) displacement experiments demonstrated a single class of specific TRH binding sites in the trout pituitary with an estimated K_d of 6.93 nM and capacity (B_{max}) of 530 fmol/mg protein. Displacement of [^{3}H]MeTRH with the native ligand (heterologous displacement) demonstrated two classes of receptors. Specifically, the two classes were differentially distributed between the two lobes of the pituitary; a small number (87 fmol/mg protein) of picomolar affinity sites in the PD and a significantly larger number (252 fmol/mg protein) of nanomolar affinity sites in the NIL. The goldfish pituitary also contained two classes of differentially distributed receptors which did not differ significantly ($p < 0.05$) in affinity or capacity when compared to the trout counterpart. Attempts to displace [^{3}H]MeTRH from trout pituitary receptors with various TRH analogs demonstrated the strict structural requirements for the receptor. Alteration of TRH at any of the three positions results in a near complete loss of receptor recognition as is evident by the relative inabilities of the studied analogs to displace radioligand from the receptor.

The trout hypothalamus contained a smaller number (9 fmol/mg protein) of nanomolar affinity sites similar to those in the NIL. It is interesting to note that the nanomolar sites appear to be restricted to tissues of neural origin (NIL and hypothalamus)

while the sites with picomolar affinities are localized on the endodermally derived PD. One would expect that in all cases, [^{3}H]MeTRH would be displaced from its receptors by greater concentrations of radiostable MeTRH; this however, is not the case with respect to the picomolar affinity PD sites. Demonstration of the higher affinity class of receptors became evident through heterologous displacement of the radioligand (TRH displacement of [^{3}H]MeTRH) which revealed both the nanomolar and picomolar affinity sits; while homologous (MeTRH) displacement indicated only the nanomolar affinity receptors. It is possible that the [^{3}H]MeTRH used in these experiments may contain small amounts of a stereoisomer produced during the tritiation proceedure which binds to the picomolar affinity receptors but is not present in the radiostable MeTRH. Furthermore, should this be the case, displacement of such a stereoisomer would likely occur with small concentrations of TRH thereby revealing this class of receptors. Therefore, it is increasingly important not to rely solely on homologous displacement analysis when investigating under these conditions.

In teleosts, the PD and NIL are directly innervated by neurosecretory fibres; therefore, neurochemicals are released directly or in proximity to target endocrine cells rather than being transported to the pituitary via the hypothalamal-hypophyseal portal system as in higher vertebrates (Peter *et al.*, 1990). The teleost NIL is extensively innervated by immunoreactive TRH (ir-TRH) neurosecretory fibres while no ir-TRH fibres are detected in the PD (Batten *et al.*, 1990). Interestingly, TRH has been shown to stimulate the release of growth hormone (Trudeau *et al.*, 1992; Wigham and Batten, 1984) and prolactin (Wigham and Batten, 1984) from the teleost PD. Additional evidence also

suggests TRH activation of thyrotropes located on the PD since intraperitoneal injection of TRH into the rainbow trout and arctic charr significantly elevates thyroxine (T_4) levels (Eales and Himick, 1988).

Since TRH stimulates hormone release from the teleost PD while no ir-TRH neurons are located there, it is necessary to consider the source of the hormone functioning there. The teleost pituitary receives blood from a hypophyseal artery which forms a primary plexus of capillaries in the neurohypophyseal tissue from which a secondary plexus forms supplying blood to the adenohypophysis (Peter *et al.*, 1990). Although no neurosecretory terminals are observed in contact with the primary capillary plexus (Peter *et al.*, 1990), it is likely that quantities of TRH are passed by diffusion from the NIL to the PD. This possibility helps explain the presence of the picomolar affinity sites on the PD and the ability of TRH to stimulate the release of various hormones from the different endocrine cell types located there.

TRH neurons in the NIL are in close proximity to groups of melanocorticotropic cells (Batten *et al.*, 1990), suggesting direct stimulation of α-MSH release. In the trout NIL, TRH actively stimulates α-MSH release *in vitro*, with a minimum required dose of 10^{-9}M and estimated ED_{50} of 1.73 X 10^{-9}M. The structural requirements of the receptor were examined by investigating the differential abilities of the various TRH analogs in the stimulation of α-MSH release from superfused NIL fragments. Of the analogs examined, only MeTRH had a pronounced effect on α-MSH secretion. The minimum effective dose and ED_{50} were 10^{-10}M and 1.5 X 10^{-8}M respectively suggesting MeTRH is an effective TRH agonist in the trout pituitary. This finding is consistent with data from

mammalian models where MeTRH is 3 to 10 times more avidly bound than the native ligand (Hinkle, 1989).

Substitution of the central histidine residue with phenylalanine produces an analog with minimal stimulatory ability, [Phe^2]TRH was only effective in stimulating α-MSH release at 10^{-6} M, while any alteration of TRH at either the amino or carboxy terminus results in near complete loss of receptor recognition. These findings are consistent with those determined by competition of 10^{-6}M [Phe^2]TRH with 10^{-9}M [^{3}H]MeTRH for binding sites on the trout pituitary where only approximately 60% of the radioligand was displaced.

The trout hypothalamus contains a single class of nanomolar affinity TRH receptors as revealed by homologous and heterologous displacement of [^{3}H]MeTRH from membrane preparation. The presence of these receptors is consistent with the distribution of ir-TRH within the teleost brain (Hamano *et al.*, 1990). TRH cell bodies in the hypothalamus are in close proximity to proopiomelanocortin (POMC), neuropeptide Y (NPY) and dopamine ß-hydroxylase (DßH) neurons (Liao *et al.*, 1991) suggesting these factors may play a role in the regulation of TRH release. Specifically, hypothalamic TRH release is stimulated by dopamine, L-dopa, and bromocryptine, a specific DA:D2 agonist (Maeda and Frohman, 1980). The function of these hypothalamic TRH receptors is not yet clear; however, they may serve to regulate the release of other neurohormones from the hypothalamus.

In conclusion, this study determined the presence of two distinct classes of pituitary TRH receptors; nanomolar sites distributed on the NIL and picomolar sites on

the PD. Furthermore, the TRH receptors display extreme structural specificity where alteration at any of the three amino acid positions results in significant reductions in receptor recognition as is evident by the decreased ability to stimulate α-MSH release from the trout NIL and displace [^{3}H]MeTRH from pituitary homogenates at a concentration of 10^{-6} M. The existence of specific TRH binding sites in the teleost hypothalamus suggests that TRH regulation of pituitary function may occur at the pre-optic/hypothalamus level.

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APPENDIX A: Determination of kinetically derived estimates of k_{-1}, k_{+1}, and K_d.

The kinetically derived dissociation rate 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 estimated on the basis of plotting ln (B_{sp}/B_o) versus time (min.) after addition of excess TRH (where B_{sp} = the specifically bound radioligand at time "t" and B_o = the specifically bound radioligand at time "0"); the slope of the resulting line is k_{-1} (min^{-1}) (Figure A1).

k_{+1} was estimated from (k_{obs} - k_{-1})/[L]; where k_{obs} is the observed rate of association and [L] is the concentration of radioligand used. k_{obs} was calculated on the basis of plotting ln $B_{eq}/(B_{eq}-B_t)$ versus time (min.) after initiation of incubation (where B_{eq} = equilibrium bound [^{3}H]MeTRH and B_t = bound radioactivity at time "t"); the slope of the resulting line is $-k_{obs}$ (M^{-1} mol^{-1}) (Figure A2).

For example, the kinetically derived K_d for the hypothalamic TRH receptor was calculated as:

$$k_{+1} = \frac{k_{obs} - k_{+1}}{[L]}$$

$$= \frac{0.039 - 0.02}{1.15\ nM}$$

$$= 1.64 \times 10^7\ M^{-1}\ mol^{-1}$$

Therefore, $K_d = k_{-1}/k_{+1}$

$= 1.21 \times 10^{-9}$ M.

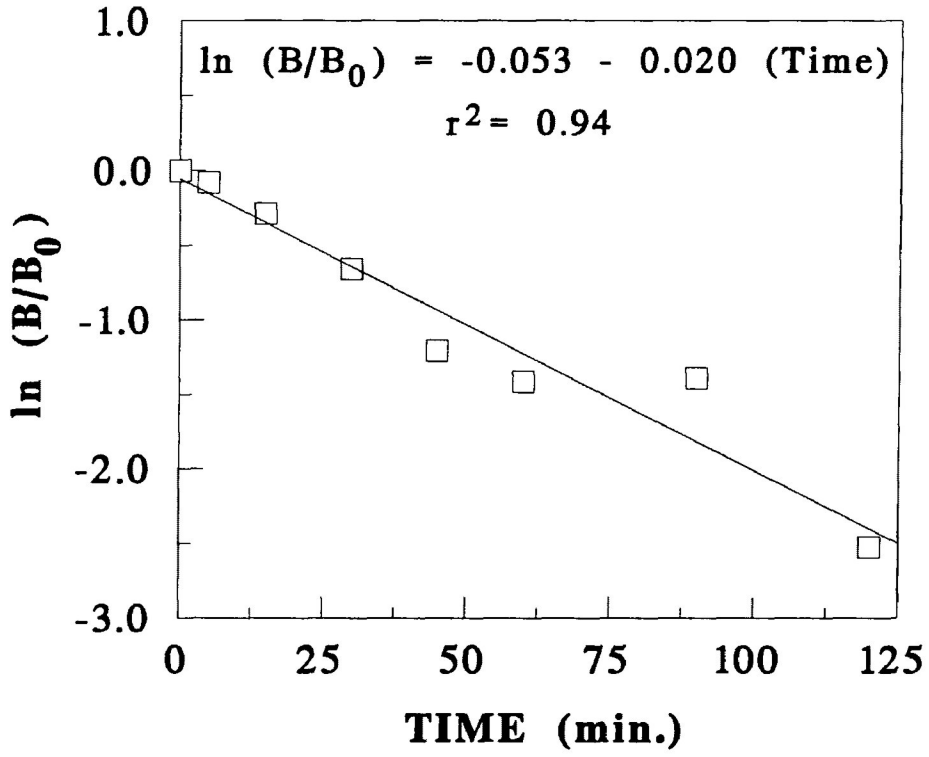


Figure A1: Determination of k_{-1} from dissociation of [^{3}H]MeTRH from trout hypothalamus data. The estimated rate of dissociation was 0.02 min^{-1}.

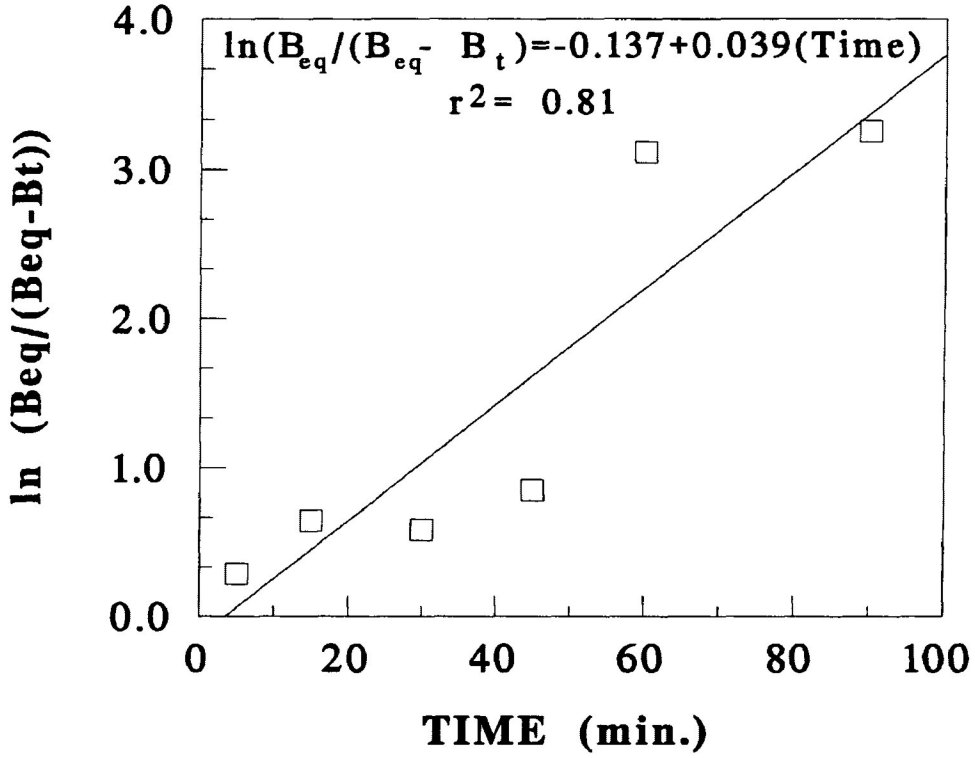
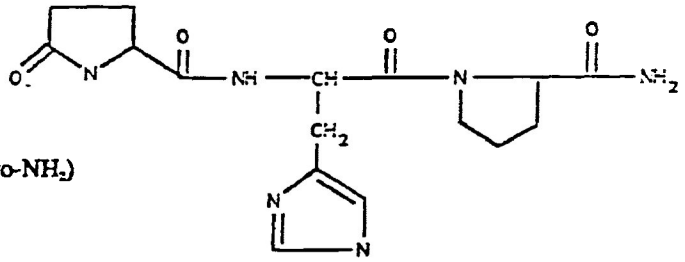
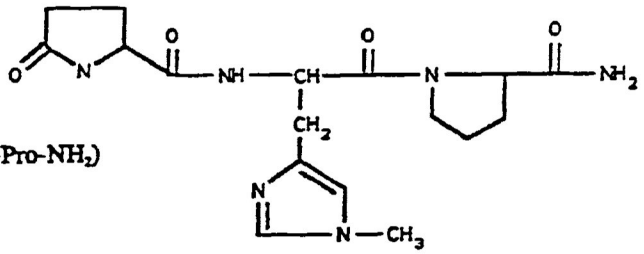


Figure A2: Determination of k_{obs} from association of [^{3}H]MeTRH to trout hypothalamus data. The estimated observed rate of association was 0.039 $M^{-1}min^{-1}$.

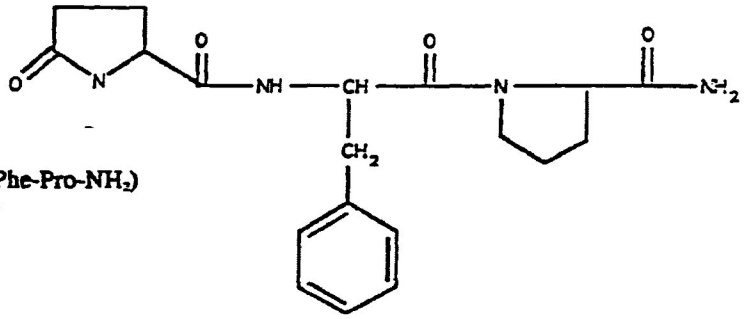
APPENDIX B: Structures of TRH and TRH-analogs used in this study



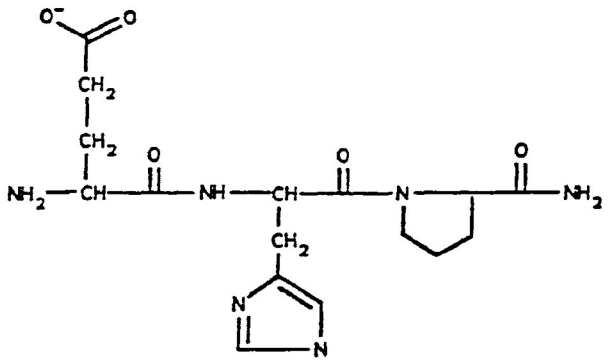
TRH: (pGlu-His-Pro-NH_2)



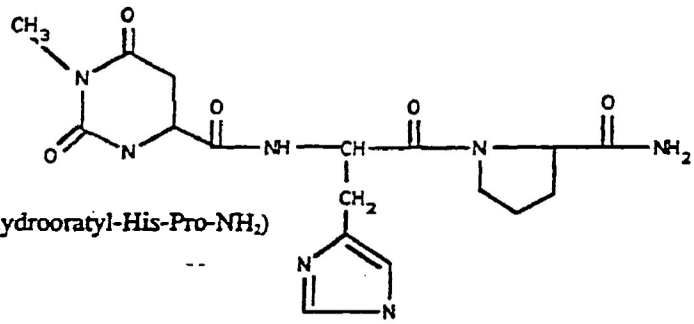
MeTRH: (pGlu-3-Me-His-Pro-NH_2)



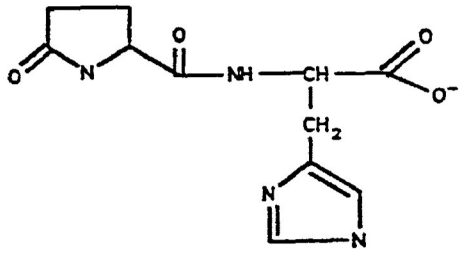
[Phe^2]TRH: (pGlu-Phe-Pro-NH_2)



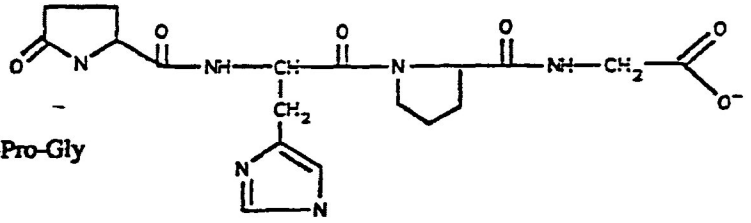
[Glu^1]TRH: (Glu-His-Pro-NH_2)



[1-Me-(S)-dihydrooratyl^1]TRH: (1-Me-(S)-dihydrooratyl-His-Pro-NH_2)



pGlu-His



TRH-Gly: pGlu-His-Pro-Gly

APPENDIX C: Probit analysis of α-MSH RIA standard curve.

The α-MSH standard curve results from the competition between radiolabeled and radiostable α-MSH for primary antibody binding sites (Figure C1). With increasing concentrations of radiostable hormone, decreased amounts of ^{125}I-radioactivity occur in the pellets.

The detection limits of the assay can be evaluated by transforming the data to probit values and plotting them against the corresponding concentration of α-MSH standard. Probit values are calculated as the amount of radioactivity bound in the presence of a specific concentration of competing hormone (B) expressed as a decimal ratio of the amount of radioligand bound in the absence of competitor (B_0). Determined hormone contents are reliable when they lie between the detection limits of the assay which correspond to the linear portion of the probit plot.

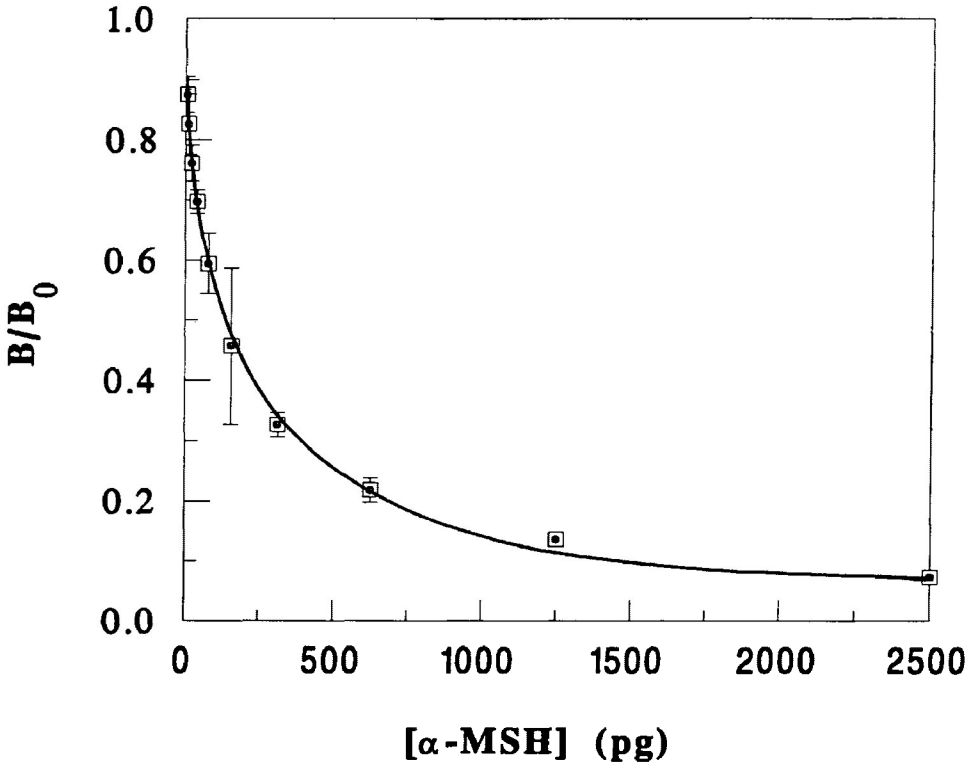
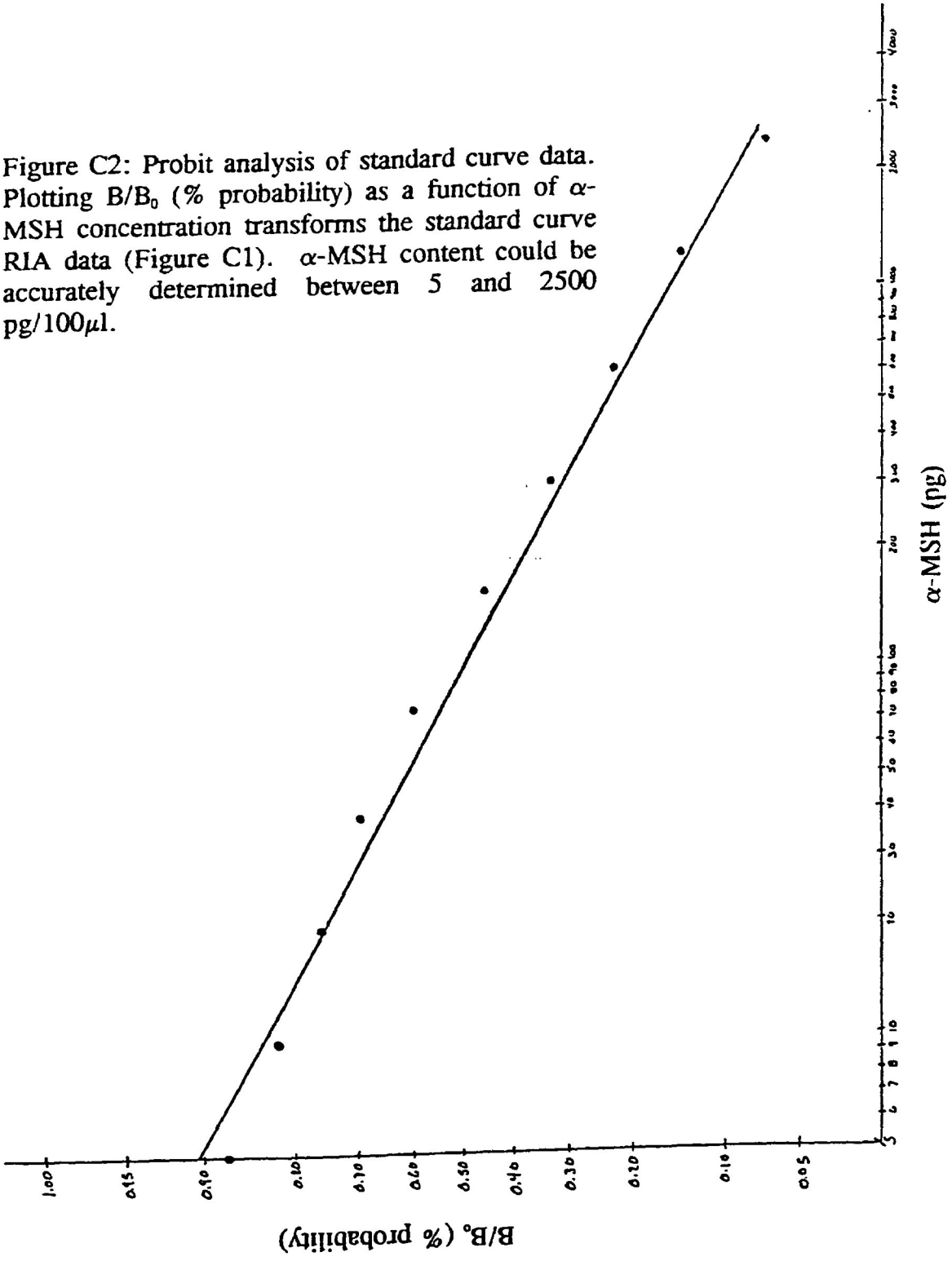


Figure C1: Sample α-MSH RIA standard curve. Radiostable α-MSH dose-dependently inhibits [^{125}I] α-MSH binding to primary antibody.

Figure C2: Probit analysis of standard curve data. Plotting B/B_0 (% probability) as a function of α-MSH concentration transforms the standard curve RIA data (Figure C1). α-MSH content could be accurately determined between 5 and 2500 pg/100μl.



APPENDIX D: Mathematical justification of ED_{50} calclation.

When a TRH pulse is administered, an α-MSH release response may or may not occur depending on the intensity of the stimulus; for example, a 10^{-10} M TRH pulse is not intense enough to ilicit an increase in α-MSH release while a 10^{-9} M TRH pulse is. The probability of a given dose eliciting an α-MSH release response can be denoted as:

1) $$P = \int_{o}^{x_o} f(x)dx$$

Since P is a unit of probability, expressed as a function of x_o (when x is small, P will be 0 and with increasing X, P increases to 1) P acts like a normal distribution function.

Therefore, for a logistic curve:

2) $P = [1 + \exp(-\alpha-\beta x)]^{-1}$, $-\infty < x < \infty$ (Govindarajulu, 1988)

3) $y = \text{logit } P = \ln(P/1-P) = \alpha + \beta x$ (Govindarajulu, 1988)

Since the values of P are based on decimal ratios of the maximal response (P = 1), the ED_{50} value will have a corresponding P value of 0.5. Substitution into equation 3 yields:

$$\begin{aligned} y &= \ln(0.5/\ 1\text{-}0.5) \\ &= \ln(1) \\ &= 0 \end{aligned}$$

Thus, the ED_{50} value occurs at the x intercept (y = 0) and is calculated from the regression equation as:

$$\begin{aligned} y &= a + bx \\ 0 &= a + bx \\ x &= -a/b \end{aligned}$$

APPENDIX E: Figures from Chapter 1 including error bars.

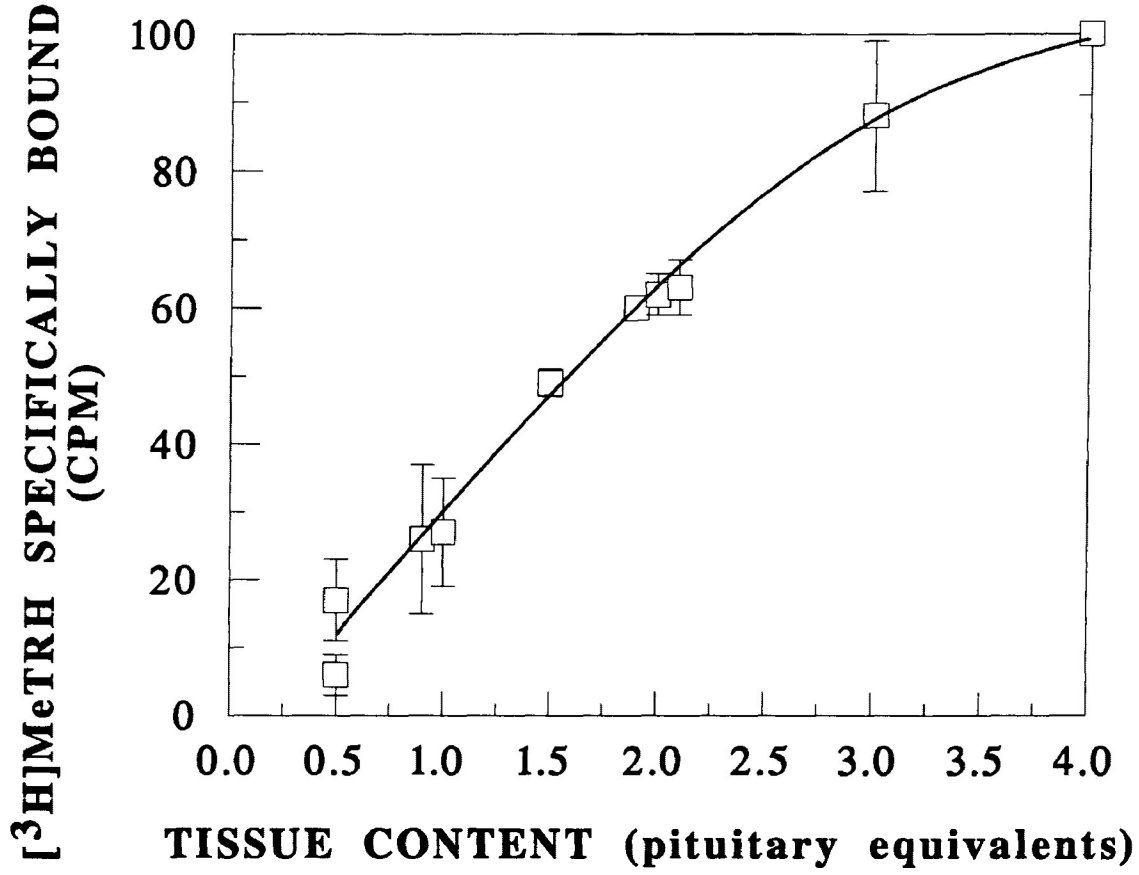


Figure E1: Specific binding (B_{sp}) of [^{3}H]MeTRH to various concentrations of washed trout pituitary membrane preparations.

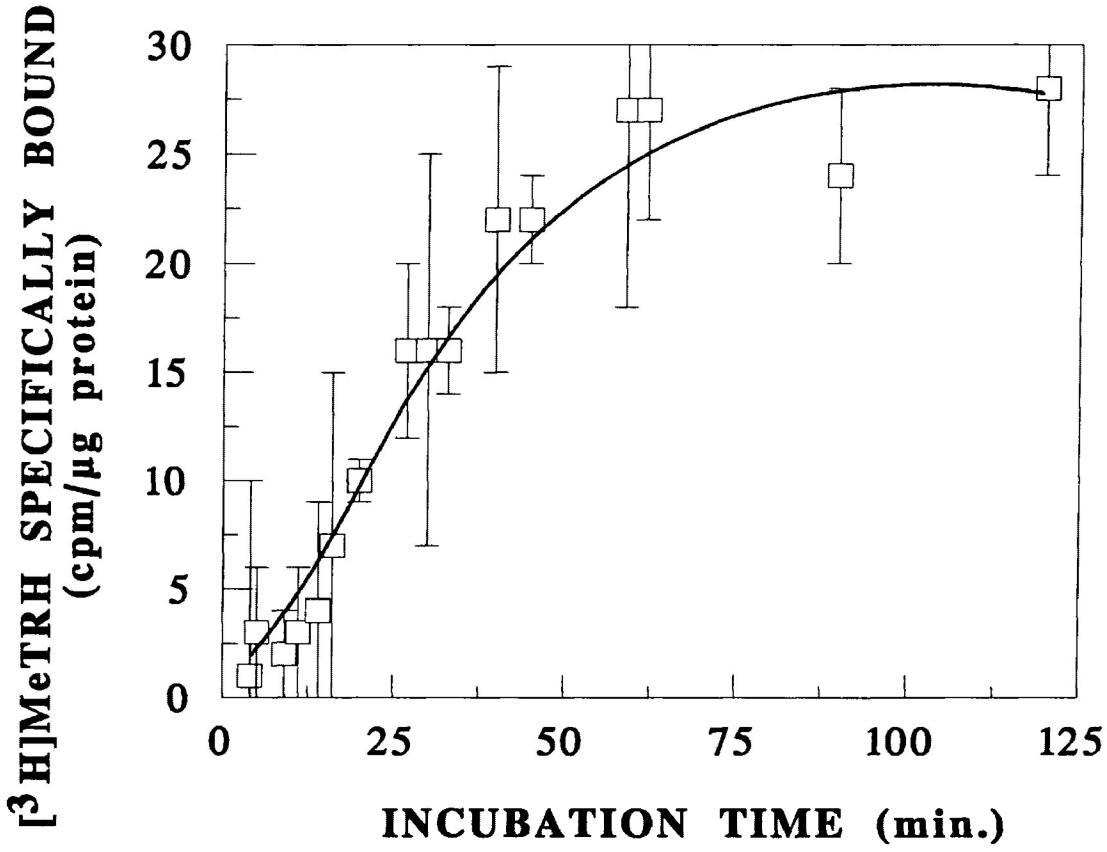


Figure E2: Association of specifically bound [^{3}H]MeTRH to washed trout pituitary resuspension increases as a function of time.

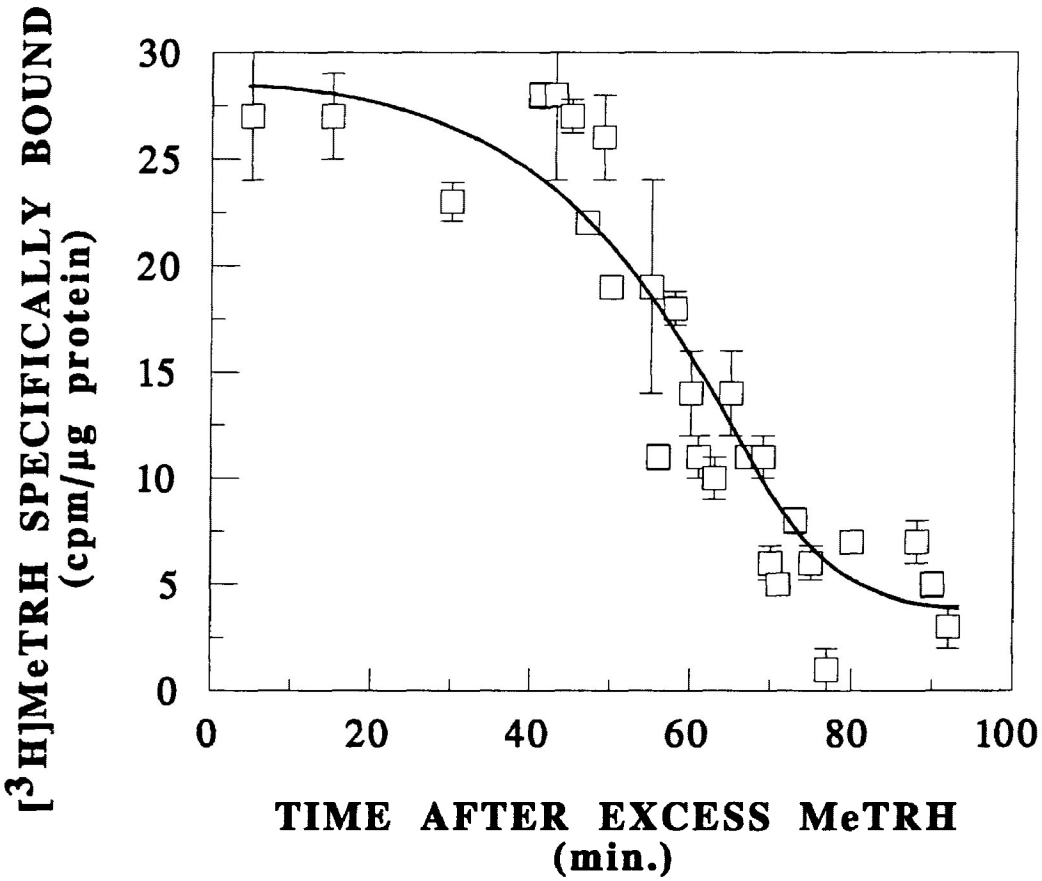


Figure E3: Dissociation of maximally bound [^{3}H]MeTRH from washed trout pituitary membrane preparation following the inclusion of excess MeTRH.

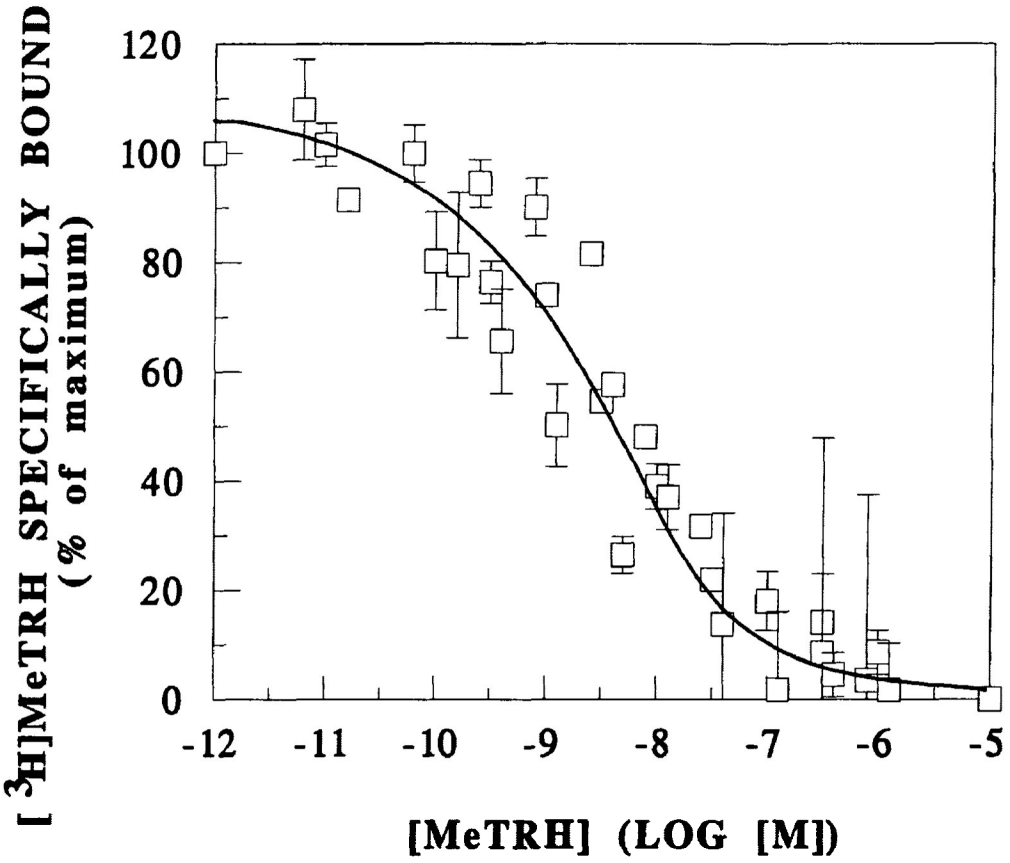


Figure E4: Homologous displacement of [^{3}H]MeTRH from trout pituitary membrane preparation.

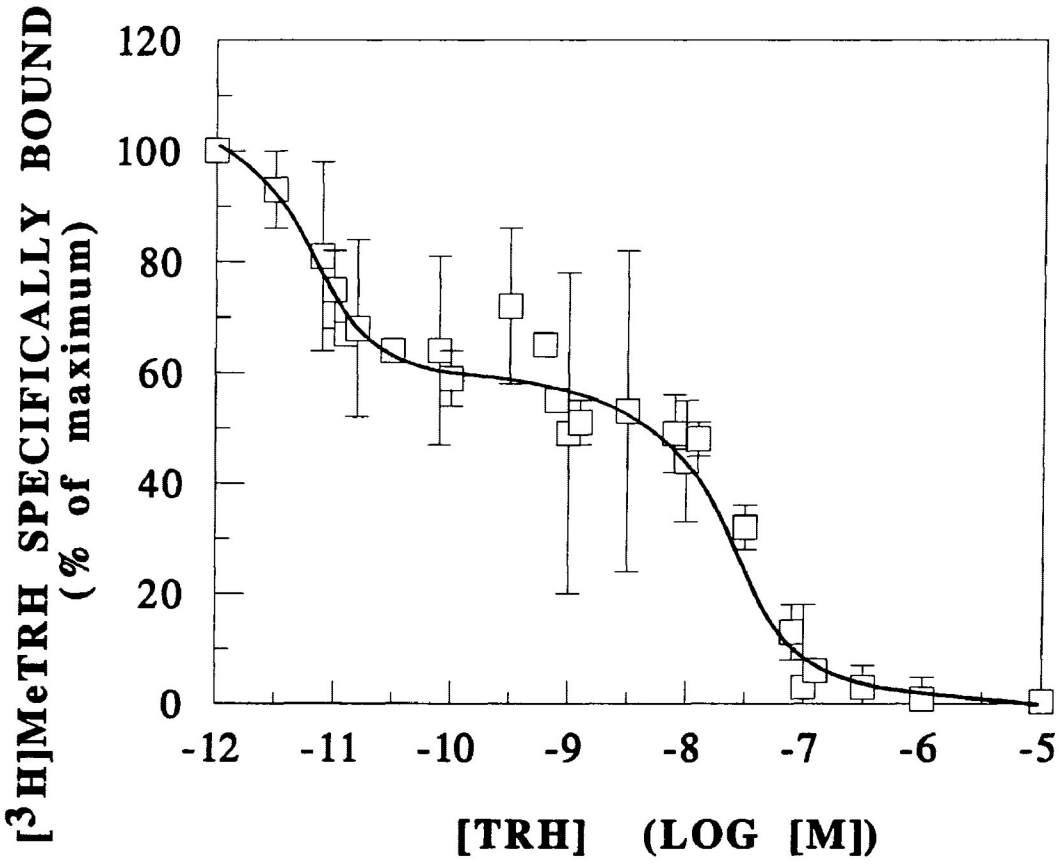


Figure E5: Displacement of [3H]MeTRH with TRH from trout pituitary membrane preparation.

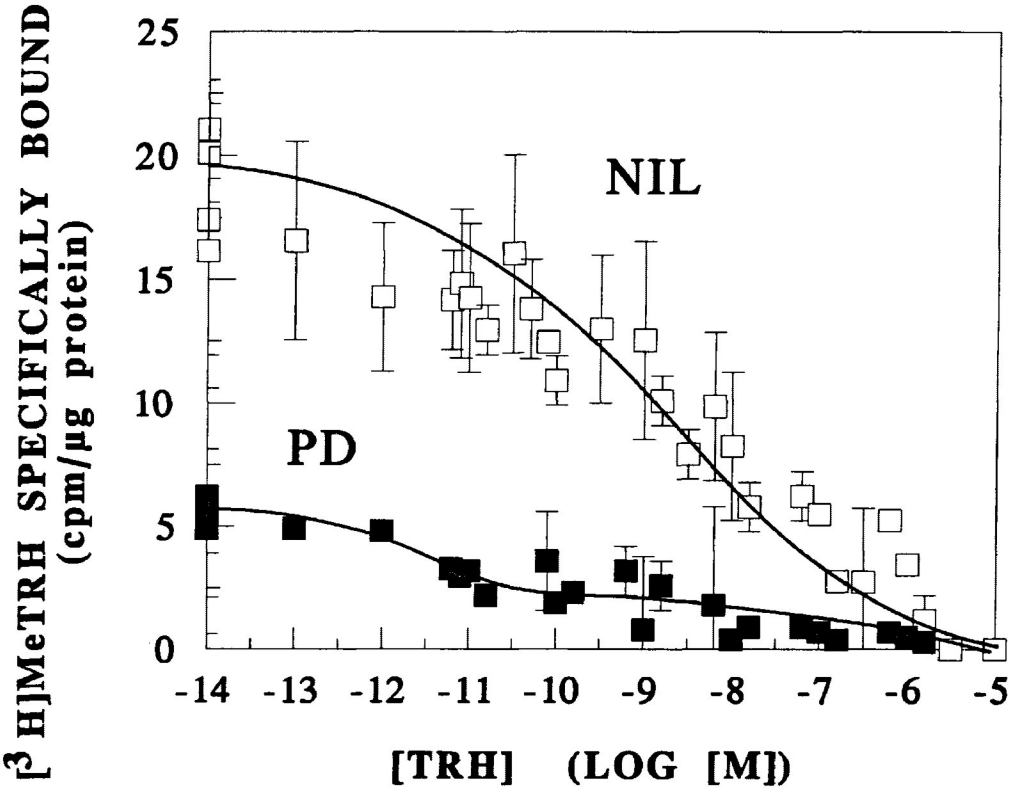


Figure E6: TRH displacement of [^{3}H]MeTRH from trout NIL and PD preparation.

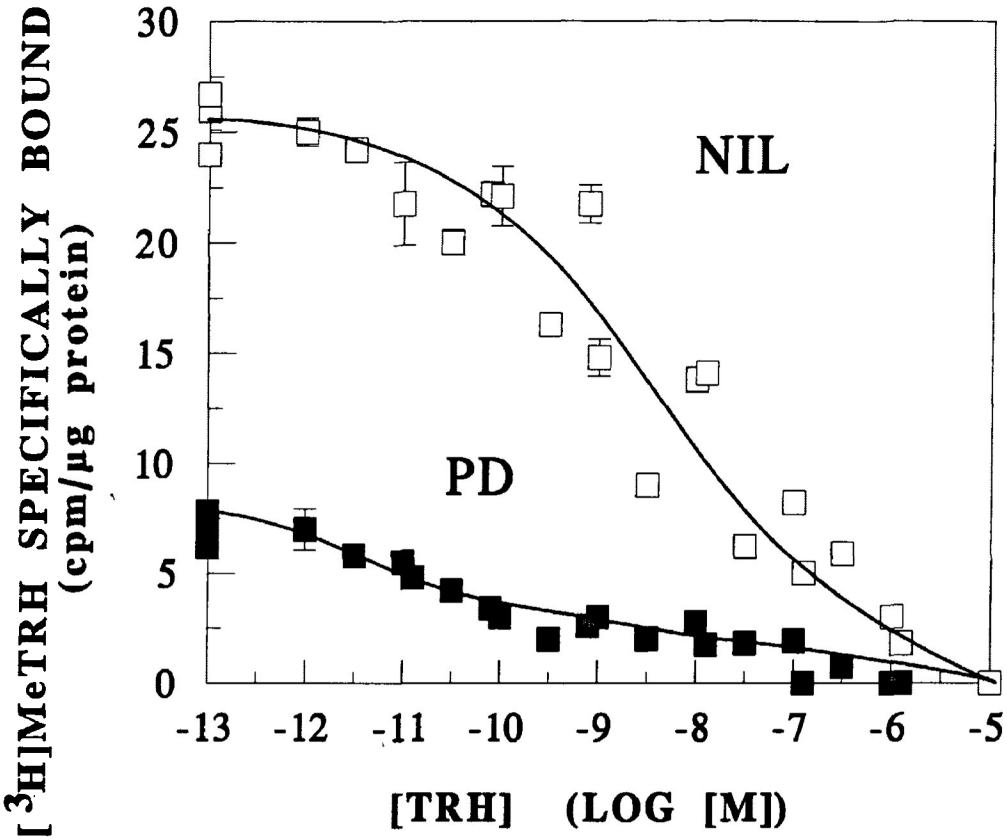


Figure E7: TRH displacement of [^{3}H]MeTRH from goldfish NIL and PD preparation.

APPENDIX F: Scatchard analysis of homologous displacement data.