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Specific Binding Sites for [^{3}H]Dexamethasone and [^{3}H]17β-Estradiol in the Hypothalamus of Juvenile Rainbow Trout, *Oncorhynchus mykiss*

By

Cyndie M. Allison

A Thesis Presented in Partial Fulfilment for the Requirements of the Degree of Master of Science

1999

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DEDICATION

This thesis is dedicated to my mother, Lillian F., and to the memory of my father, Stephen J. Allison.

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CHAPTER 1

General Introduction

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Overview:

In bony fishes such as the rainbow trout, *Oncorhynchus mykiss*, glucocorticoid (GC) and estrogenic hormones are associated with a variety of physiological functions. Most notable are effects of GCs which allow the animal to respond to stressful events (Donaldson, 1981; Clearwater and Pankhurst, 1997; Stouthart *et al.*, 1998) and the actions of estrogens in the hormonal control of reproduction (Donaldson, 1973; Arcandhoy and Benson, 1998; Arukwe and Goksoyr, 1998). Circulating levels of GCs and estrogens are regulated largely by activation of the hypothalamic-pituitary-interrenal (HPI) axis (Donaldson, 1981; Stouthart *et al.*, 1998), and the hypothalamic-pituitary-gonadal (HPG) axis (Fostier *et al.*, 1983; Blazquez *et al.*, 1998; Kah *et al.*, 1997), respectively. In the case of the HPI axis, the initial stimulus for biosynthesis and release of cortisol, the primary plasma GC, is corticotropic releasing hormone (CRH). Neurons of hypothalamic origin project their axons to pituitary target cells, corticotrophs (Peter *et al.*, 1990). CRH released onto corticotrophs stimulates release of adrenocorticotropic hormone (ACTH) into circulation (Baker *et al.*, 1996; Stouthart *et al.*, 1998). ACTH serves as the final signal in this pathway by stimulating interrenal tissue to synthesize and release cortisol into circulation (Chester-Jones *et al.*, 1969). In turn, the level of plasma GCs may provide a feedback signal to the HPI axis, via activation of hypothalamic glucorticoid receptors (GRs).

HPG axis activity regulates the production of 17β-estradiol (E_2), the principle plasma estrogen synthesized and released by ovarian follicles (Donaldson, 1973; Arukwe and Goksoyr, 1998). Hypothalamic neurons that release gonadotropic releasing hormone (GnRH) project their axons onto pituitary gonadotrophs, whereby GnRH induces the pituitary to synthesize and release gonadotropic hormones (GtHs) into circulation. In teleosts, GtH I is the predominant

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gonadotropic hormone prior to sexual maturity while GtH II is released after maturation is complete (Suzuki *et al.*, 1988; Breton *et al.*, 1997; Breton *et al.*, 1998). In the mature fish, the ovaries serve as the primary site for the GtH II-induced synthesis and release of E_2 (Campbell, 1980), whereas the interrenals serve as an additional, albeit limited, source of E_2 in male and female animals before and after sexual maturity (Hoar, 1969; Kime *et al.*, 1980; Fitzpatrick *et al.*, 1993). In teleosts, plasma E_2 levels may provide a feedback signal to the HPG axis via activation of hypothalamic E_2 receptors (ERs).

Peripheral Function of Cortisol in Teleosts:

Cortisol supports multiple physiological functions in fish, including maintenance of metabolic homeostasis (van der Boon *et al.*, 1990) as well as regulation of hydromineral balance (Chester-Jones *et al.*, 1969). Cortisol biosynthesis occurs very early in fish development; for example, elevated plasma levels have been observed in the rainbow trout 2 to 4 days prior to hatching (Yeoh *et al.*, 1996; Stouthart *et al.*, 1998). Subsequently, changes in GC secretion occur daily (Audet and Claireaux, 1992) and seasonally (Audet and Claireaux, 1992; McLeese *et al.*, 1994; Shrimpton and McCormick, 1998) as well as in response to stressors (Donaldson, 1981; Clearwater and Pankhurst, 1997; Stouthart *et al.*, 1998). A rise in the level of plasma cortisol during spawning (Chester Jones *et al.*, 1969; Pickering and Christie, 1981; Scott *et al.*, 1983) and migration (McLeese *et al.*, 1994) are associated with an increase in the plasma level of glucose consistent with glucose mobilization required for increased energy expenditures (Zelnick and Goldspink, 1981; Nielsen *et al.*, 1994). Cortisol also assists migratory fish, including various salmonids and eels, in their adaptation to seawater entry by altering the structure and function of specific osmoregulatory organs (Shrimpton and McCormick, 1998). Specifically, smolting

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salmonids are characterized by a seasonal rise in plasma cortisol levels which are correlated with increases in the number of chloride cells and levels of Na^+/K^+ ATPase activity in gill tissue, and coincident with reductions in plasma Na^+ levels (McLeese *et al.*, 1993). Prolonged exposure to elevated plasma levels of cortisol due to chronic stress, however, can lead to an impaired ability to regulate ion transport across gill tissue, thereby impeding successful seawater entry (Chester-Jones *et al.*, 1969).

Peripheral Function of E_2 in Teleosts:

In salmonids, sexual development leading to spawning is characterized by rhythmic changes in circulating E_2 levels (Campbell *et al.*, 1980; Scott *et al.*, 1983; Fitzpatrick *et al.*, 1986). A complex interaction of hormones (Donaldson, 1973; Trudeau *et al.*, 1991; Arukwe and Goksoyr, 1998) and neurotransmitters (Kah *et al.*, 1992; Trudeau *et al.*, 1993; Saligaut *et al.*, 1998) coordinates HPG axis activity during the reproductive cycle and modulates release of E_2 from ovarian follicles facilitating gonadal development (Hoar, 1969; Blazquez *et al.*, 1998), oocyte maturation (Scott *et al.*, 1983; Fitzpatrick *et al.*, 1986), and the synthesis of vitellogenin, a precursor of yolk proteins produced by the liver (Ho, 1987; Flouriot *et al.*, 1997; Arukwe and Goksoyr, 1998; Davail *et al.*, 1998; Rinchard *et al.*, 1998). High levels of plasma E_2, associated with the onset of sexual maturation, promote the utilization of carbohydrate reserves (Soengas *et al.*, 1993; Washburn *et al.*, 1993) and reduce chloride cell number and blood osmolality (Coimbra *et al.*, 1992). These E_2 -induced alterations help to physiologically maintain anadramous fish in freshwater where spawning occurs. During this period an elevated level of plasma E_2 provides a negative feedback signal on HPG axis activity inhibiting subsequent GtH release. However, as oocytes mature just prior to ovulation, plasma E_2 levels drop while GtH levels rise indicating

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disinhibition of HPG axis activity in coho salmon (Fitzpatrick *et al.*, 1986) and rainbow trout (Scott *et al.*, 1983; Breton and Sambroni, 1996).

Steroid Influence on Vertebrate CNS Function:

Steroid hormones such as glucocorticoids (Rostene *et al.*, 1995; Holsboer and Barden, 1996) and estrogens (Pilgrim and Hutchinson, 1994) have the ability to function as neuromodulators of vertebrate central nervous system (CNS) functioning. While many steroids, particularly ring A-reduced metabolites of progesterone and glucocorticoids, rapidly influence neuronal activity within milliseconds after receptor activation and have a short duration of activity (Majewska, 1987; Paul and Purdy, 1992; Mellon, 1994; Lambert *et al.*, 1995), the onset of steroid-induced genomic changes in target cell bioactivity is more delayed, but has prolonged effects (McEwen, 1994). Unlike "classical" neurosteroids that are synthesized *de novo* in the CNS (Mellon, 1994), cortisol and E_2 biosynthesis originates outside the CNS. Specifically, there is a separation of the peripheral and central actions of E_2 since it originates as a metabolite of peripherally synthesized testosterone which is secreted into circulation by testicular tissue in sexually mature males and from ovarian follicles in mature females (Kagawa *et al.*, 1982).

GCs in the Mammalian CNS:

In most vertebrates examined, activation of the hypothalamic-pituitary-adrenal (HPA) axis, a basic survival mechanism, occurs upon exposure to stressors (Selye, 1950; McEwen, 1994). In humans, physical (exercise, injury, etc.) or perceived (fear) stressors initiate an increase in hypothalamic CRH secretion stimulating the release of ACTH into circulation which ultimately enhances adrenal GC secretion (Selye, 1950). Since they are lipid-soluble, circulating GCs are able to cross the blood:brain barrier and provide a feedback signal to regulate HPA axis activity at

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multiple levels (De Kloet, 1991). Periphally produced GCs have also been shown to impact directly on brain function in mammals. For example, a stress-induced elevation of adrenal GC secretion coincides with a reduction in the number of $GABA_A$ receptors in the rat hypothalamus (Weizman *et al.*, 1990). This allows for excitatory activity in CRH-producing neurons that are inhibited by GABA binding (Herman and Cullinan, 1997). Prolonged exposure to elevated GCs during chronic physical and psychological stress, however, is thought to be a contributing factor to impaired brain function. In two nonhuman primates, the African green monkey (Uno *et al.*, 1989) and the Rhesus monkey, chronic GC exposure during confinement (maximum plasma cortisol: 70 μg/dl) and as a result of GC administration (dexamethasone treated; 23.3 to 51.4 μg/dl versus control; 18.1 to 41.8 μg/dl) results in a 20 to 30% loss of hippocampal CA3 and CA4 pyramidal neurons in both adult and fetal animals (Uno *et al.*, 1994). Damage in this brain region interferes with HPA axis control of GC secretion by interrupting the feedback inhibition of hypothalamic CRH release (DeKloet *et al.*, 1993; Uno *et al.*, 1994).

GCs also influence neurosecretory activity in the mammalian CNS. In the rat brain, for example, acute high doses of GCs (corticosterone and dexamethasone) inhibit DA release from hippocampal neurons and serotonin (5HT) release from neurons in the hippocampus and prefrontal cortex, whereas chronic high doses of corticosterone increase DA and 5HT metabolism in the prefrontal cortex (Inque and Koyama, 1996). By contrast, GCs inhibit release of noradrenaline (NOR) in the rat brain (Wolfovitz *et al.*, 1995).

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GCs in Teleost CNS:

GCs provide a feedback signal to HPI axis activity controlling subsequent GC secretion. Balm and Pottinger (1998) report that increases in plasma ACTH and cortisol levels occur within 3 hours after the onset of a stress event. ACTH returns to a baseline level, however, much sooner than concurrent reductions in plasma cortisol levels. The authors suggest that HPI axis feedback is due to inhibition of ACTH secretion from the pituitary rather than a reduced sensitivity of GRs to ACTH by the interrenals. Similarly, endogenous (Fryer and Peter, 1977; Sumpter *et al.*, 1986; Balm and Pottinger, 1998) and synthetic (Pickering *et al.*, 1987) GCs have the ability to modulate ACTH secretion. GCs have also been identified as regulators of neurotransmitter activity in teleosts where elevated plasma GCs levels associated with a primary stress response coincide with a surge in catecholamine (i.e. DA, NOR, and adrenaline (ADR)) synthesis and release in rainbow trout (Reid *et al.*, 1996).

E_2 in the Mammalian CNS:

In mammals E_2 is considered to be an important regulator of CNS development and neuronal function (for review see Pilgrim and Hutchinson, 1994). The presence of E_2 in the brain occurs through the metabolism of testosterone of systemic origin. The conversion of testosterone into E_2 (aromatization) is catalyzed by a group of enzymes known as the aromatase system, a member of the cytochrome P450 superfamily, whose activity in the rat brain is positively regulated by the plasma level of androgens (Roselli *et al.*, 1984; Roselli and Resko, 1987). Aromatization of testosterone within the hypothalamus is crucial for sexual differentiation of developing nuclei (Simerly and Young, 1991; Pilgrim and Hutchinson, 1994) and occurs during critical periods of CNS development and organization (Arnold and Gorski, 1984). In rats (Gorski *et al.*, 1980; Wise

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et al., 1981; Arnold and Gorski, 1984; Kolbinger *et al.*, 1991) and humans (Hofman and Swaab, 1989) E_2 is responsible for a variety of site-specific effects on the female and male brain such as differences in the numbers, size, and morphology of neurons. To illustrate, female rats possess fewer (Tobet *et al.*, 1989; Beyer *et al.*, 1991; Yuri and Kawata, 1994) and smaller hypothalamic dopaminergic neurons than in males (Kolbinger *et al.*, 1991). As well, there is more rapid development of their neuronal processes in this region due to a limited exposure to E_2 in female rats (Reisert *et al.*, 1989). Similarly, GnRH-secreting neurons in the female hypothalamus contain greater numbers of dendritic processes as a result of reduced exposure to E_2 than typically found in males rats (Raisman and Field, 1973). While much of the differentiation and organization of the neuronal circuitry occurs in mammals during early development, many of these pathways continue to be modified as a result of E_2 exposure during sexual maturation. For example, Yuri and Kawata (1994) showed that elevated plasma E_2 levels influence DA activity in hypothalamic neurons of adult female rats by increasing DA content in periventricular preoptic neurons coincident with decreased content in medial preoptic neurons. E_2 also has the ability to influence the secretory capacity of dopaminergic neurons in adult female rats. Limited CNS exposure to E_2 promotes an increased DA output in females compared to males (Di Paolo *et al.*, 1985) from the hypothalamus. These differences in neuronal circuit activity may also contribute to sex-based variations in neurohormonal regulation by the hypothalamus. For example, in humans, basal ACTH levels are typically lower in females than males contributing to a more robust response to stress (i.e. higher cortisol levels) by the HPA axis in males (Murphy, 1991).

E_2 and the Teleost CNS:

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E_2 has a primary role in modulating HPG axis activity in teleosts. In maturing female rainbow trout a rising plasma E_2 level provides a positive feedback signal to the HPG axis, stimulating GtH I secretion from the pituitary as a result (Prat *et al.*, 1996). In sexually mature salmonids, by contrast, chronically elevated plasma E_2 levels prior to ovulation act as a negative feedback signal on the HPG axis culminating in a decline of plasma E_2 levels (autoregulation) (Crim *et al.*, 1981; Mayer *et al.*, 1991; Scott *et al.*, 1992) with a concurrent increase in GtH II secretion (Prat *et al.*, 1996; Saligaut *et al.*, 1998) during the period of ovulation. However, E_2 may not have the ability to directly modulate its own secretion rate. To illustrate, the administration of E_2 has been shown to stimulate the synthesis and release of DA from hypothalamic neurons in rainbow trout (Saligaut *et al.*, 1992; Saligaut *et al.*, 1998) which serve, in part, to inhibit GnRH release (Peter *et al.*, 1990). In contrast, E_2 administration increases NOR production in hypothalamic neurons that stimulate GnRH release (Saligaut *et al.*, 1992) whereas NOR inhibits the pituitary from releasing GtH (Peter *et al.*, 1990).

Glucocorticoid and E_2 Receptors:

Steroid hormones such as cortisol and E_2 elicit changes in the bioactivity of their target cells by binding and activating their respective receptors. Traditionally, steroid receptors have been shown to reside in the cytosol coupled to a heat shock protein (hsp90) in the absence of hormone. Lipid soluble steroids readily cross the plasma membrane of the cell and are able to bind their respective receptors in a unimolecular interaction. Once bound by hormone, the heat shock protein dissociates and the newly "activated" receptor-steroid complex is translocated to the nucleus where it binds a specific region on the DNA molecule, referred to as a hormone response element (HRE) (Lewin, 1992), and alters genomic activity in the target cell (Funder, 1993).

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Ultimately, the binding of GC and E_2 to their receptors throughout the HPA and HPG axis, respectively, is required to initiate the feedback signals regulating circulating levels of these hormones.

Sex steroid receptors depart from the traditional model in that they have been shown to reside in the nucleus prior to activation by hormone (King and Greene, 1984; Welshons *et al.*, 1984). The subcellular location of glucocorticoid receptors (GR) has been difficult to resolve (Gustafsson *et al.*, 1986; Lee *et al.*, 1992; Pottinger *et al.*, 1994; Knoebl *et al.*, 1996). Much of the evidence for GRs is based on cytosolic binding activity with limited support for a nuclear locus (Porthe-Nibelle and Lahlou, 1984; Chakraborti *et al.*, 1987; Brink *et al.*, 1992; McLeese *et al.*, 1994; Weisbart *et al.*, 1994).

Glucocorticoid Receptors:

While GRs are distributed throughout the mammalian body, they are expressed in greatest numbers in the hippocampus (Sapolsky *et al.*, 1984; DeKloet *et al.*, 1993; Smith *et al.*, 1994; van Steensel *et al.*, 1996) and throughout HPA axis. GR activity in the rat hippocampus is primarily involved in cognitive functions such as the storage of spatial information and the organization of behavioural responses, but it also plays a fundamental role in modulation of GC secretion whereby a stress-induced levels of GCs stimulate a rise in hippocampal GR numbers facilitates the feedback signal on HPA axis activity (DeKloet *et al.*, 1993). GR distribution within the rat HPA axis, however, is nonuniform with the highest density occurring in the pituitary compared to the hypothalamus (Sapolsky *et al.*, 1984; DeKloet *et al.*, 1993; Smith *et al.*, 1994).

Recently the binding parameters such as the affinity of the GR for cortisol (K_d = 4.54 $\pm$

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0.06 n*M*) and the number of GRs (B_{MAX} = 25.40 ± 2.20 fmol/mg protein) have been characterized for adult chinook salmon (*Oncorhynchus tshawytscha*) whole brain preparations (Knoebl *et al.*, 1996). In addition, Lee *et al.* (1992) have demonstrated the sensitivity of rainbow trout GRs to exogenous GC treatment. The administration of dexamethasone (1.5 mg/kg body weight), a synthetic GR agonist, resulted in a rapid reduction in the number (82.3 ± 2.5 to 20.6 ± 10.5 fmol/mg protein) and affinity (K_d = 1.56 ± 0.19 to 4.4 ± 0.5 n*M*) of GRs in whole brain preparations within 3 hours which persisted for 24 hours. To date, GR mRNA has been localized throughout the forebrain of mature female and immature rainbow trout with significant representation within CRH-releasing neurons of the hypothalamus (Teitsma *et al.*, 1997; Teitsma *et al.*, 1998) and anterior pituitary (Teitsma *et al.*, 1998). However, GR binding characteristics in specific regions of the salmonid HPI axis have not been identified prior to this investigation.

E_2 Receptors:

The fine tuning of E_2 secretion throughout the reproductive cycle is regulated via ERs throughout the HPG axis. The presence of ERs in the CNS of the rat has been well documented, especially for regions such as the hypothalamus (Simerly and Young, 1991; Blaustein, 1992; Brown *et al.*, 1995) and preoptic area (Blaustein, 1992; Brown *et al.*, 1995). The ER in the hypothalamus has been shown to be sensitive to the level of circulating E_2, decreasing in number in response to elevated levels of the hormone (Brown *et al.*, 1995).

Evidence for ERs in the teleost CNS has been indicated by the localization of ER mRNA in the forebrain and hypothalamus of rainbow trout (Salbert *et al.*, 1991; Teitsma *et al.*, 1998). In addition, a number of investigations (Davis *et al.*, 1977; Kim *et al.*, 1978; Morrell and Pfaff, 1978; Fine *et al.*, 1990; Linard *et al.*, 1996) implicate the preoptic area as a possible site for E_2

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modulation of the HPG axis in the rainbow trout due to the abundance of ER-positive cells, primarily on dopaminergic neurons in this region (Linard *et al.*, 1996). E_2-containing cells have also been identified in the thalamus (Kim *et al.*, 1978; Fine *et al.*, 1990), telencephalon (Kim *et al.*, 1978; Morrell and Pfaff, 1978; Fine *et al.*, 1990), hypothalamus (Davis *et al.*, 1977; Kim *et al.*, 1978; Morrell and Pfaff, 1978; Fine *et al.*, 1990) and pituitary (Kim *et al.*, 1978; Morrell and Pfaff, 1978; Fine *et al.*, 1990) providing indirect evidence for the presence of ERs. While these studies suggest that ERs are distributed throughout the teleost brain-pituitary axis, an investigation into their relative abundance and binding affinities in these regions has yet to be undertaken.

Coordinated Actions of GCs and E_2:

Up to this point, GCs and E_2 and their respective HP axes have been presented as independent systems. However, there is an interaction in terms of the impact that each has on mutual hypothalamic-pituitary axes and receptor activities. For example, elevated plasma E_2 prolongs activation of the HPA axis the female rat resulting in higher basal plasma corticosteroid levels for a period of 3 weeks (Burgess and Handa, 1992). Specifically, GRs in the female rat undergo a 20 percent decrease in the pituitary during peak circulating levels of estrogen (Turner, 1990), whereas GRs numbers in the hypothalamus increase (Ferrini and De Nicola, 1991). Similarly, E_2 contributes to the regulation of HPA axis activation of the teleosts. The administration of E_2 facilitates an increase in both basal and stress-induced plasma cortisol level in immature trout, but not in mature fish already possessing naturally elevated plasma E_2 levels (Pottinger *et al.*, 1996).

Similarly, GCs extend their influence to the activities of the HPG axis. Estacio *et al.*

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(1996) demonstrated that stress drastically reduces luteinizing hormone (LH) secretion from the pituitary of female rats. This diminished LH secretion occurs through the enhancement of the feedback signal via a stress-induced increase in the number of ERs in the hypothalamus. In female rainbow trout stress directly impacts on vitellogenesis, the production of yolk proteins (Ho, 1987; Clearwater and Pankhurst, 1997; Contrerassanchez *et al.*, 1998). While elevation of plasma cortisol appears to have no direct influence on E_2 secretion, it reduces both cytosolic and nuclear GR numbers in the liver and enhances (> 33%) plasma E_2 binding to sex steroid binding globulin (Pottinger and Pickering, 1990).

While the actions of GCs and E_2 appear to be interdependent in their influence on the activities of the HPA and HPG axes, the timing of when this interaction commences has yet to be elucidated. All of the aforementioned investigations have been conducted on animals that have attained sexual maturity. My investigations, however, utilize only juvenile rainbow trout which have no prior exposure to elevated plasma levels of gonadal hormones. In this case, I hope to establish the characteristics for GC and E_2 binding sites in the hypothalamus prior to the onset of sexual maturation.

Criteria for defining receptor sites:

The next two chapters of this thesis detail my investigation of receptors sites in the rainbow trout according to established criteria for defining such sites. According to Laduron (1984), receptor binding assays should elucidate all of the following characteristics :

a) The subcellular distribution of the receptor site demonstrating whether it is located within the cytosolic, nuclear, or both components of the cell;

b) the presence of a finite number of receptors as indicated by a saturable level of ligand

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binding;

c) a high affinity of binding (K_d in the nanomolar range) to the receptor by the ligand;

d) reversible ligand binding as indicated by the association and subsequent dissociation of the receptor:ligand complex;

e) tissue specificity of the receptor as indicated by a linear relationship between specifically bound ligand and the amount of tissue utilized;

f) ligand binding which is readily displaced by structurally related compounds;

g) the correlation of *in vitro* drug affinitity to drug potency *in vivo*; and

h) the regional distribution of the receptor throughout the animal;

As this investigation does not include the two latter elements, I will be referring to the putative GRs and ERs in the hypothalmus of the juvenile rainbow trout as "binding sites" throughout the Discussion section of this thesis.

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CHAPTER 2

Allison, C.M. and Omeljaniuk, R.J. (1998). Specific binding sites for

[^{3}H]dexamethasone in the hypothalamus of juvenile rainbow trout,

Oncorhynchus mykiss.

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ABSTRACT

Indirect evidence suggests that glucocorticoid hormones may act through cellular receptors to play a neuromodulatory role in the teleost CNS. We now report our findings on the use of [^{3}H]dexamethasone (DEX) to identify hypothalamic glucocorticoid receptors (GRs) in juvenile rainbow trout, *Oncorhynchus mykiss*.

Hypothalamic cytosol was incubated with [^{3}H]DEX under various experimental paradigms with incubations terminated by addition of dextran-coated charcoal; following immediate centrifugation, a sample of bound [^{3}H]DEX (supernatant) was collected and assessed for ^{3}H content. [^{3}H]DEX binding was tissue dependent between 0.5 and 2.0 hypothalamus equivalent per tube (1.0 to 4.7 mg protein, respectively). Specific binding (B_{SP}) increased with time for 1.5 hr and remained relatively constant for an additional 2.5 hr; the calculated association rate constant was 2.23×10^8 $M^{-1} \times min^{-1}$. Equilibrium B_{SP} was dissociated by addition of a 5000 molar excess cortisol with an accompanying $t_{1/2}$ of 1.25 hr and dissociation rate constant of 0.553 min^{-1}. B_{SP} was saturable with a calculated equilibrium K_d and B_{MAX} of 1.22 n*M* and 296 fmol/mg protein, respectively. B_{SP} was displaced under equilibrium conditions by the corticosteroids, but to a lesser extent by the mineralocorticoid, estrogen, and progestin. The rank order of potency for [^{3}H]DEX displacement was DEX > cortisol >> corticosterone > triamcinolone = 11-deoxycortisol >> aldosterone > progesterone >>> 17β-estradiol. These properties of specifically bound [^{3}H]DEX indicate the presence of a GR, similar to the mammalian cytosolic GR, in the hypothalamus of juvenile rainbow trout.

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INTRODUCTION

Glucocorticoid hormones (GCs) support multiple functions in vertebrates. In bony fishes (teleosts), for example, GCs maintain metabolic homeostasis (van der Boon *et al*., 1990) and participate in the regulation of hydromineral balance (Chester Jones *et al*., 1969). In salmonids, such as the rainbow trout (*Oncorhynchus mykiss*), elevated levels of plasma cortisol, the major circulating GC, are observed during spawning (Chester Jones *et al*., 1969; Pickering and Christie, 1981; Scott *et al*., 1983), migration (McLeese *et al*., 1994), exposure to environmental stressors (Pottinger *et al*., 1994) and during immune responses (Pickering, 1984; Maule and Schreck, 1991). Elevated GC levels are associated with altered glucocorticoid receptor (GR) activity in a variety of tissues including gill epithelia (Sandor *et al*., 1984; Chakraborti *et al*., 1987; Maule and Schreck, 1991; McLeese *et al*., 1994; Weisbart *et al*., 1994), leukocytes (Maule and Schreck, 1991), and liver (Pottinger, 1990; Lee and Struve, 1992; Pottinger *et al*., 1994). To illustrate, cortisol levels increase in response to confinement stress leading to a decreased affinity (increased K_d) and a reduction in the number (downregulation) of the cytosolic GRs in coho (*O. kisutch*) gill epithelia (Maule and Schreck, 1991) and rainbow trout liver (Pottinger *et al*., 1994). During subsequent acclimation, both affinity and capacity of cytosolic GRs have been shown to return to preconfinement levels in coho salmon (Maule and Schreck, 1991) gill epithelia and in the liver of rainbow trout (Pottinger, 1990; Pottinger *et al*., 1994). Cortisol's ability to alter GR activity in a vast array of tissue types can lead to changes in bioactivity in the affected target cells. Ultimately, an effective mechanism of HPI axis activation is required enabling the animal to modulate the release of cortisol during periods of prolonged exposure to stressful events.

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Enhanced GC secretion in teieosts proceeds from activation of the hypothalamic-pituitary-interrenal (HPI) axis (Donaldson, 1981) whereby neurosecretory cells originating in brain regions, including the hypothalamus, directly innervate specific target cells in the pituitary (Peter *et al.*, 1990) allowing the release of corticotropic releasing factor from the hypothalamus to regulate adrenocorticotropic hormone (ACTH) secretion from individual cells (corticotropes) in the pituitary (Fryer and Peter, 1977; Peter *et al.*, 1990). ACTH consequently stimulates the release of GCs (mainly cortisol) from interrenal tissue (Chester Jones *et al.*, 1969). Until recently, GRs in the teleost pituitary have been regarded as the main targets for GC modulation of the HPI feedback loop, since evidence for cortisol's influence on HPI activity has been limited to its effects on ACTH secretion (Fryer and Peter, 1977; Sumpter *et al.*, 1986). However, binding sites for GCs such as cortisol (Knoebl *et al.*, 1996), DEX (Lee *et al.*, 1992) and triamcinolone acetonide (TA) (Knoebl *et al.*, 1996) have been found in the salmonid brain suggesting a higher level for GC control of the HPI axis. For example, Lee *et al.* (1992) found that cytosolic GRs numbers in rainbow trout whole brain were reduced after the administration of DEX, a synthetic GC with a high affinity for the GR.

Although classically regarded as hormones with actions peripheral to the CNS, GCs are also being considered as modulators of neuronal activity within the CNS of mammals (for review see Holsboer and Barden, 1996). To illustrate, elevated levels of plasma GCs in the rat modulate regional brain synthesis and release of dopamine (DeKloet *et al.*, 1993; Rostene *et al.*, 1995; Inque and Koyama, 1996), noradrenaline (Pacak *et al.*, 1995; Wolfovitz *et al.*, 1995), and serotonin (DeKloet *et al.*, 1993; Inque and Koyama, 1996). In the teleost model, Reid *et al.* (1996) reported that elevated plasma cortisol levels were correlated with the release of

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noradrenaline and adrenaline from sympathetic neurons innervating interrenal tissue in the rainbow trout. In the context of whole animal responsivity to stress, these sequential changes in GC and neurotransmitter release are coincidental with increases in metabolic activities (Reid *et al.*, 1996) preparing an animal for a "fight or flight". These data suggest that catecholaminergic neurons in the teleost brain, including hypothalamus, may be sensitive to HPI axis activity by acting as targets for GCs.

GRs are thought to be widely distributed throughout the mammalian brain and have been identified in the rat hippocampus (Sapolsky *et al.*, 1984; DeKloet *et al.*, 1993; Smith *et al.*, 1994; van Steensel *et al.*, 1996) and hypothalamus (Sapolsky *et al.*, 1984; Smith *et al.*, 1994), as well as in the pituitary (Sapolsky *et al.*, 1984; Smith *et al.*, 1994). However, there is little direct evidence (Knoebl *et al.*, 1996) for the presence, distribution, and pharmacological resolution of the GR in a site-specific manner in the teleost brain.

We report the existence and pharmacological characteristics of specific [^{3}H]DEX binding sites in the hypothalamus of juvenile rainbow trout as a site for feedback regulation of HPI activity. This investigation is part of our ongoing research into the coordinated actions of GCs and other classes of steroids as neuromodulators in the teleost brain.

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METHODS AND MATERIALS

Animals. Juvenile rainbow trout (Rainbow Springs Hatchery, Thamesford, Ontario, Canada) were raised and maintained at the Lakehead University Aquatic Animal Research Facility in aquaria supplied with flow-through, dechlorinated water at simulated ambient temperature (5 to 16° C, annual range) and photo period (8 to 16 hr, annual range). Fish were fed daily *ad libitum* with commercial trout pellets (Ziegler trout pellets, Thunder Bay Co-Op). Prior to handling, fish were anaesthetized with tricaine methanesulfonate (MS 222, 0.05g/litre; Syndel Laboratories, Vancouver, BC) and killed by spinal transection posterior to the medulla oblongata.

Tissue Preparation. Hypothalami were excised and immediately transferred to a polystyrene tube, immersed and stored in liquid nitrogen, until assayed. The hypothalamus was defined as the region ventral to the thalamus and posterior to the telencephalon. Dissection commenced at the optic tract and extended posteriorly to the nucleus diffusus lobi inferioris (Peter and Gill, 1975). Preliminary experiments showed no obvious difference in [^{3}H]DEX binding to fresh or frozen rainbow trout hypothalamus preparations (Allison, 1997). Consequently, experiments were performed using frozen tissue. This receptor assay was based on modification of methods by Smith *et al*. (1994) and Weisbart *et al*. (1994). Briefly, pooled hypothalami were thawed in ice-cold TEDMS buffer (10 m*M* Tris; 1 m*M* EDTA; 1 m*M* dithiothreitol; 20 m*M* sodium molybdate; 250 m*M* sucrose; 10% (v/v) glycerol; pH 7.4), homogenized on ice using 10 strokes of a motor-driven Teflon-glass homogenizer (0.125 mm clearance) and centrifuged at 1500g for 20 min. The supernatant was decanted and centrifuged for 1 hr at 40,000g to obtain the cytosolic (supernatant) fraction. All steps were carried out at 0-4°C unless stated otherwise.

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A 100μl aliquot of cytosol was incubated with a 100μl volume of [^{3}H]DEX (3.3 n*M*) (39.22 Ci/mmol; NEN-Dupont, Boston, MA) in the presence (nonspecific binding, NSB) or absence (total binding, B_O) of 100μl of a 1000-fold molar excess of radioinert cortisol (Sigma Chemicals, St. Louis, MO) in a final volume of 0.3 ml in 12x75 mm glass tubes. Incubations were terminated by the addition of 300μl of dextran-coated activated charcoal (DCC) (0.125% dextran, 1.25% charcoal in TEDMS buffer (w/v); Sigma Chemicals, St. Louis, MO), vortexed, incubated for 10 min, and centrifuged at 1500g for 10 min. A 400μl aliquot of the supernatant containing bound [^{3}H]DEX was placed in a 6 ml scintillation vial in combination with 4 ml of liquid scintillation cocktail (Ready Safe, Beckman Instruments Inc., Fullerton, CA), mixed, and allowed to incubate overnight in the dark. Sample radioactivity was determined by liquid scintillation spectroscopy using a Beckman LS-6500 liquid scintillation spectrometer (50% counting efficiency).

Specific Radioreceptor Assays

Tissue dependence of [^{3}H]dexamethasone binding to hypothalamus cytosol preparations. Various dilutions of hypothalamus cytosol were incubated, in triplicate experiments, for 16 hr with [^{3}H]DEX in the presence (NSB) or absence (B_O) of 1000-fold molar excess cortisol. One hypothalamus equivalent per tube was subsequently used since it gave a significant signal in the linear range of tissue dilutions.

Association of [^{3}H]dexamethasone to hypothalamus cytosol preparations. In triplicate experiments, trout hypothalamus cytosol preparations were incubated for various intervals with [^{3}H]DEX in the presence (NSB) or absence (B_O) of 1000-fold molar excess cortisol prior to termination.

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Dissociation of [^{3}H]dexamethasone from hypothalamus cytosol preparations. Hypothalamus cytosol was incubated with [^{3}H]DEX for 2 hr to equilibrium binding. After the addition of 5000-fold molar excess radioinert cortisol, tubes were vortexed and incubated for various intervals prior to termination. Experiments were performed in triplicate.

Saturation analysis. In triplicate experiments, hypothalamus cytosol was incubated for 16 hr with various concentrations (0.06 to 43.5 n*M*) of [^{3}H]DEX in the presence (NSB) or absence (B_O) of 1000-fold molar excess cortisol prior to termination.

Competitive Displacement Analysis. In triplicate experiments, hypothalamus cytosol was incubated with [^{3}H]DEX in the presence of competitors at various concentrations. Reaction mixtures were incubated for 2 hr prior to termination. Competitors were purchased from Sigma Chemicals (St. Louis, MO) and represented major steroid groups such as glucocorticoids (cortisol and corticosterone), mineralocorticoids (aldosterone), estrogens (17β-estradiol), and the progestins (progesterone). Synthetic glucocorticoids (dexamethasone and triamcinolone) and nonglucocorticoid (11-deoxycortisol) competitors were also utilized.

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

Data Analysis. Specific binding (B_{SP}) was calculated as the difference between total (B_O) and nonspecific (NSB) binding. Standard error of the mean (SEM) = $(\sigma^2_{Bsp} + \sigma^2_{NSB})^{1/2}$ (Hulme and Birdsall, 1992). First order transformations of kinetic data were analysed according to Bennett and Yamamura (1985) to determine association (k_{+1}), dissociation (k_{-1}) rate constants, and equilibrium dissociation constant (K_d). k_{-1} was calculated from the linear regression analysis of log

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B_{SP} versus time where k_{-1} = slope of the line. 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 free radioligand. k_{obs} was calculated from the plot of ln(B_{eq}/B_{eq}- B_{SP}) versus time where Beq = the level of binding at steady state and the slope of the line = k_{obs}. The kinetically derived K_d (k_{-1}/k_{+1}) and the maximum number of binding sites (B_{MAX}) from the saturation analysis was calculated according to Scatchard (1949). Maximum specific binding (B_{SP}) was calculated as the difference between B_O (competitor absent) and NSB at 1000 molar excess of cortisol. Competitive binding data are presented as the percent of maximum B_{SP} displaced by competitor. Half-maximal inhibitory concentration (IC_{50}) values for each competitor was estimated from the plot of ln (P/1.0-P) versus competitor concentration. P values are the decimal ratios of maximal B_{SP} (P = 1.0) (Hulme and Birdsall, 1992). Scatchard (1949) analysis was performed on data from competitive binding assays to provide estimates of K_d.

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RESULTS

Tissue dependence of [^{3}H]dexamethasone binding to hypothalamus cytosol preparations. B_{SP} of [^{3}H]DEX to rainbow trout hypothalamus cytosol preparations increased linearly between 0.5 and 2.0 hypothalamus equivalents per tube (1.0 to 4.7 mg protein) (FIG. 1). One hypothalamus equivalent per tube was used in subsequent experiments.

Association of [^{3}H]dexamethasone to hypothalamus cytosol preparations. B_{SP} of [^{3}H]DEX to hypothalamus cytosol preparations increased slowly to reach equilibrium after a 1.5 hr incubation and remained stable for at least 4.0 hr (FIG. 2). Maximum B_{SP} decreased slightly between 3 to 24 hr incubation (Allison, 1997). Pooled data from three independent experiments were used to estimate (Bennett and Yamamura, 1985) $k_{+1} = 2.23 \times 10^8\ M^{-1} \times \text{min}^{-1}$.

Dissociation of [^{3}H]dexamethasone from hypothalamus preparations. Equilibrium bound [^{3}H]DEX rapidly dissociated from trout cytosol preparations after the addition of a 5000-fold molar excess of radioinert cortisol. Pooled data from three independent experiments was used to estimate (Bennett and Yamamura, 1985) the dissociation rate constant (k_{-1}) of 0.553 min^{-1} with an estimated half life ($t_{1/2}$) of 1.25 hr (FIG. 3). The kinetically derived equilibrium dissociation constant, K_d (k_{-1}/k_{+1}), was $2.48 \times 10^{-9}\ M$.

Saturation Analysis. Saturable binding activity was demonstrated for [^{3}H]DEX binding to trout hypothalamus cytosol. Scatchard analysis revealed a linear relationship ($r^2 = 0.96$) suggestive of a single class of high-affinity ($K_d = 1.22 \pm 0.2$ n*M*; n= 3) and low-capacity ($B_{MAX} = 296 \pm 64.9$ fmol/mg protein; n=3) binding sites (FIG. 4).

Competitive Displacement Analysis. DEX was a stronger competitor ($IC_{50} = 10.7$) for binding sites than the endogenous hormone, cortisol ($IC_{50} = 30.7$). Corticosterone, triamcinolone (TA),

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and 11-deoxycortisol (11-DOC) had similar competitive abilities, but had IC_{50} values two orders of magnitude higher than dexamethasone. Aldosterone, 17β-estradiol and progesterone were not effective competitors (Table 1). Rank order of potency was dexamethasone > cortisol >> corticosterone > TA = 11-DOC >> aldosterone > progesterone >>> estrogen (FIG. 5).

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DISCUSSION

Our data demonstrate the existence of a single class of saturable, high-affinity [^{3}H]DEX binding sites in the hypothalamus of juvenile rainbow trout indicative of steroid hormone receptors. The equilibrium dissociation constant of [^{3}H]DEX (Scatchard analysis, $K_d = 1.22 \pm 0.2$ n*M*; kinetic analysis, $K_d = 2.48 \pm 0.2$ n*M*) is somewhat higher than cytosolic K_d values reported for [^{3}H]TA in salmonid tissues such as whole brain (Knoebl *et al.*, 1996), gill, spleen, and kidney (Maule and Schreck, 1991), but 4-5 times lower than cytosolic assays utilizing [^{3}H]cortisol in salmonid whole brain (Knoebl *et al.*, 1996) and liver preparations (Pottinger, 1990; Pottinger *et al.*, 1994). By comparison, our findings are consistent with cytosolic binding affinities for [^{3}H]TA in rainbow trout and eel gill preparations (Sandor *et al.*, 1984; Maule and Schreck, 1991), but higher than for [^{3}H]DEX in rat hypothalamus (Smith *et al.*, 1994). There also seems to be variability in the maximum level of binding sites for specific tissue types reported between mammalian and teleost models. We report a B_{MAX} value (296 $\pm$ 64 fmol/mg protein) similar to that of rat (Sapolsky *et al.*, 1984; Dhabhar *et al.*, 1993) and mouse (Luttge *et al.*, 1989) whole brain cytosol. In teleosts, however, basal (nonstressed) B_{MAX} values reported for salmonid whole brain preparations (Knoebl *et al.*, 1996) are an order of magnitude lower than that of peripheral tissues such as gill (Sandor *et al.*, 1984; Chakraborti *et al.*, 1987) and liver (Chakraborti and Weisbart, 1987; Pottinger, 1990). While our reported B_{MAX} value is consistent with of mammals, it may reflect only unoccupied GRs that are available for binding. In the absence of a DCC pretreatment to remove endogenous cortisol from the cytosol, actual B_{MAX} values may be higher than represented in this assay. However, preliminary experiments using a DCC pretreatment failed to demonstrate an increase in [^{3}H]DEX binding (Allison and Omeljaniuk, unpublished data). In

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addition, we chose not to use the pretreatment based on evidence by Emadian *et al.* (1986) which stated that DCC pretreatment can produce a 3- to 6-fold loss in binding affinity of GRs for [^{3}H]DEX in mouse brain cytosol.

The variability for reported K_d and B_{MAX} values between brain and peripheral tissues may be associated with differences in plasma cortisol levels associated with the animal's exposure to stressors, level of sexual maturity (Pickering and Christie, 1981), or seasonal cortisol fluctuations (McLeese *et al.*, 1994) at the time of sampling. Sumpter *et al.* (1986) demonstrated that the rise in plasma cortisol levels can occur within minutes after the onset of stress. Concurrent r eductions in GRs number (up to 60%) are discernable within 24 hr after such an event (Pottinger, 1990; Pottinger *et al.*, 1994), lasting up to 3 days for acute stressors (Weisbart *et al.*, 1987; Pottinger, 1990) and up to 7 days during chronic events (Pottinger *et al.*, 1994) before GR numbers begin to return to physiological levels.

The putative GR in the cytosol of the rainbow trout hypothalamus demonstrates selective binding for a variety of ligands. The ability of various steroids to compete with [^{3}H]DEX for these binding sites is shown in FIG. 5. The synthetic GR agonist, DEX, was shown to be more effective (IC_{50} = 10.7 n*M*) than endogenous cortisol (IC_{50} = 30.7 n*M*) in its ability to displace the specifically bound, radiolabelled ligand (Table 1). DEX, a nonmetabolizable GC, is reported to have a binding higher affinity for the cytosolic GR than endogenous cortisol in salmonid tissues such as gill epithelia (Sandor *et al.*, 1984; Chakraborti *et al.*, 1987), liver (Pottinger, 1990), and whole brain preparations (Knoebl *et al.*, 1996). Our findings that DEX is a more effective competitor than TA (a synthetic GC with a high affinity for the GR) is in agreement with assays on brook trout gill (Chakraborti *et al.*, 1987) and rainbow trout liver (Pottinger, 1990) where

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either cortisol or DEX was used as the radioligand and/or competitor. However, the specificity of the GR differs slightly in cytosolic assays on chinook whole brain preparations (Knoebl *et al.*, 1996) and rainbow trout gill tissue (Sandor *et al.*, 1984) when TA was used as radioligand and/or competitor. In these assays, TA was shown to be a more effective competitor than DEX. We found that aldosterone, a mineralocorticoid with a high affinity for GRs in mammals, was not an effective competitor for [^{3}H]DEX binding. This is consistent with the fact that in teleosts there has been no evidence for maintenance of hydromineral balance by aldosterone, a function which is assumed to be regulated by GCs (Chester Jones *et al.*, 1969).

In mammals GCs are well known mediators of many activities, both in the CNS and peripherally, and have been associated with a complex array of endocrine and physiological activities. These activities are of particular importance to salmonids during periods of chronic stress (i.e. spawning and migration) when target tissues become exposed to the effects of prolonged plasma cortisol elevations. The importance of cortisol can be seen not only in its capacity to directly alter bioactivity in an array of tissue types, but also in its influence on the synthesis of other hormones. For example, elevated plasma GC levels have the capacity to modulate the reproductive development of trout by limiting gonadal steroid production (Pickering *et al.*, 1987; Carragher *et al.*, 1989; Carragher and Sumpter, 1990).

In conclusion, we have demonstrated evidence for GRs in the hypothalamus of rainbow trout that is consistent with the classical steroid receptor model. The existence of GC-binding sites in the salmonid CNS suggests an important mechanism where hormone-sensitive target sites can influence the feedback loop of the HPI axis. GC regulation of HPI activity has been demonstrated in brown trout by Pickering *et al.* (1987) where orally administered DEX was

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shown to be an effective suppressor of ACTH release, thus limiting the rise in plasma cortisol associated with a stress response. However, the level at which DEX was able to influence the feedback loop was not elucidated. Furthermore, it may be possible that GR levels in the salmonid CNS are subject to modulation by other hormones in a manner similar to that seen in mammals (Schneider and Shyamala, 1985), enabling the cell to alter its sensitivity to elevated GC levels by reducing (down-règulating) the number of GRs available.

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FIG. 1. Specific binding (B_{SP}) of [^{3}H]dexamethasone to hypothalamus cytosol preparations of juvenile rainbow trout. B_{SP} is the difference between binding in the absence (total binding; B_O) and presence (nonspecific binding; NSB) of 1000 molar excess cortisol. A linear relationship between B_{SP} and tissue content was observed for 0.5- 2.0 hypothalamus equivalents. Values are means (n= 4, ± SEM) from three independent experiments (●, ■, ▲).

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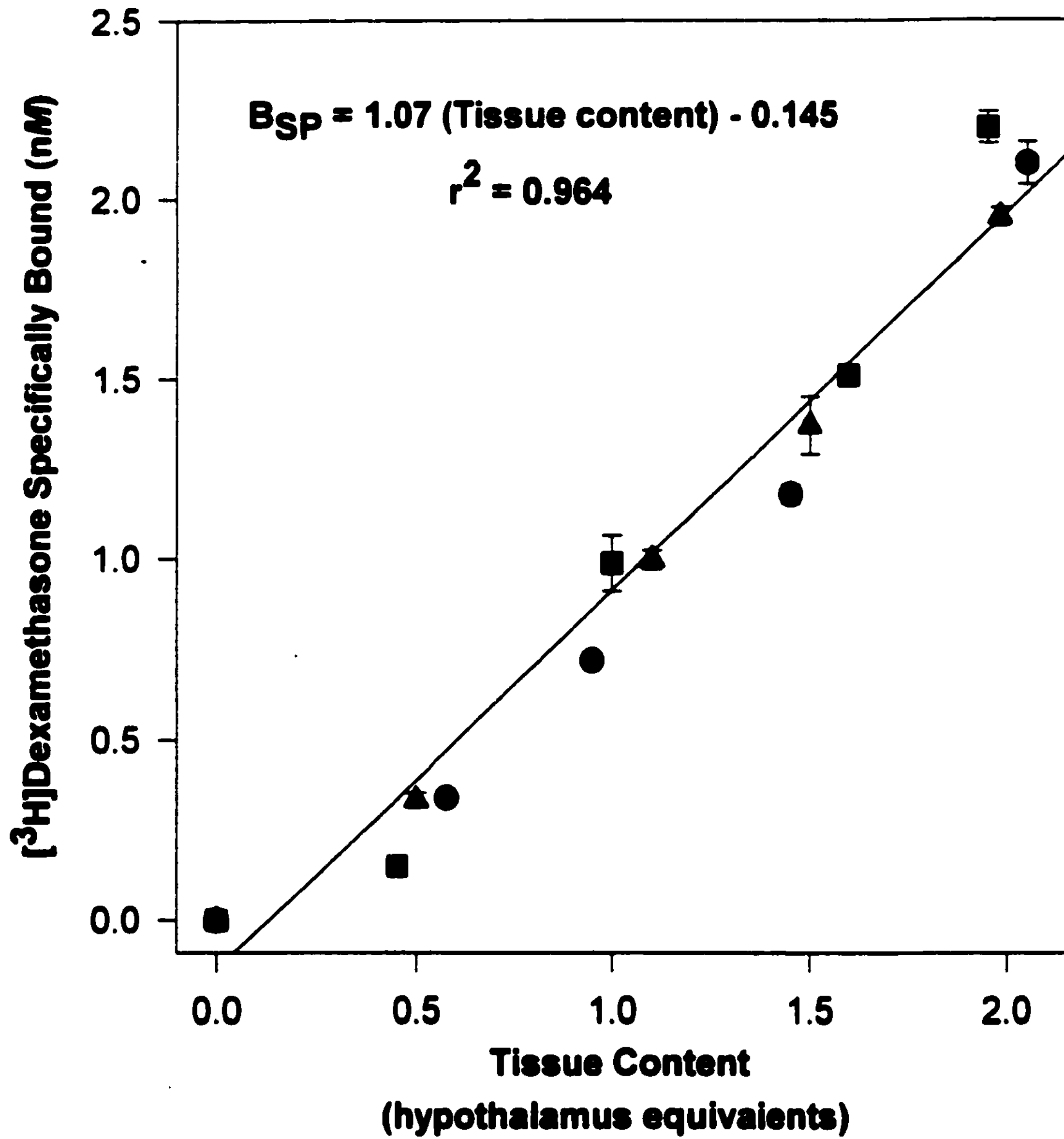

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FIG. 2. Specific binding (B_{SP}) of [^{3}H]dexamethasone (n*M*) to hypothalamus cytosol preparations of juvenile rainbow trout as a function of time. B_{SP} is the difference between binding in the absence (total binding; B_O) and presence (nonspecific binding; NSB) of 1000 molar excess cortisol. Values are means (n= 4, ± SEM) from three independent experiments (●, ■, ▲). Inset: ln (B_{eq}/B_{eq}-B_{SP}) as a function of time. Relationship (r^2=0.93) was used to determine ln (B_{eq}/B_{eq}-B_{SP}) = 1.19 (time) + 0.15 which was used to determine association rate constant (k_{+1}) as 2.23 x 10^8 M^{-1} x min^{-1} (Bennett and Yamamura, 1985).

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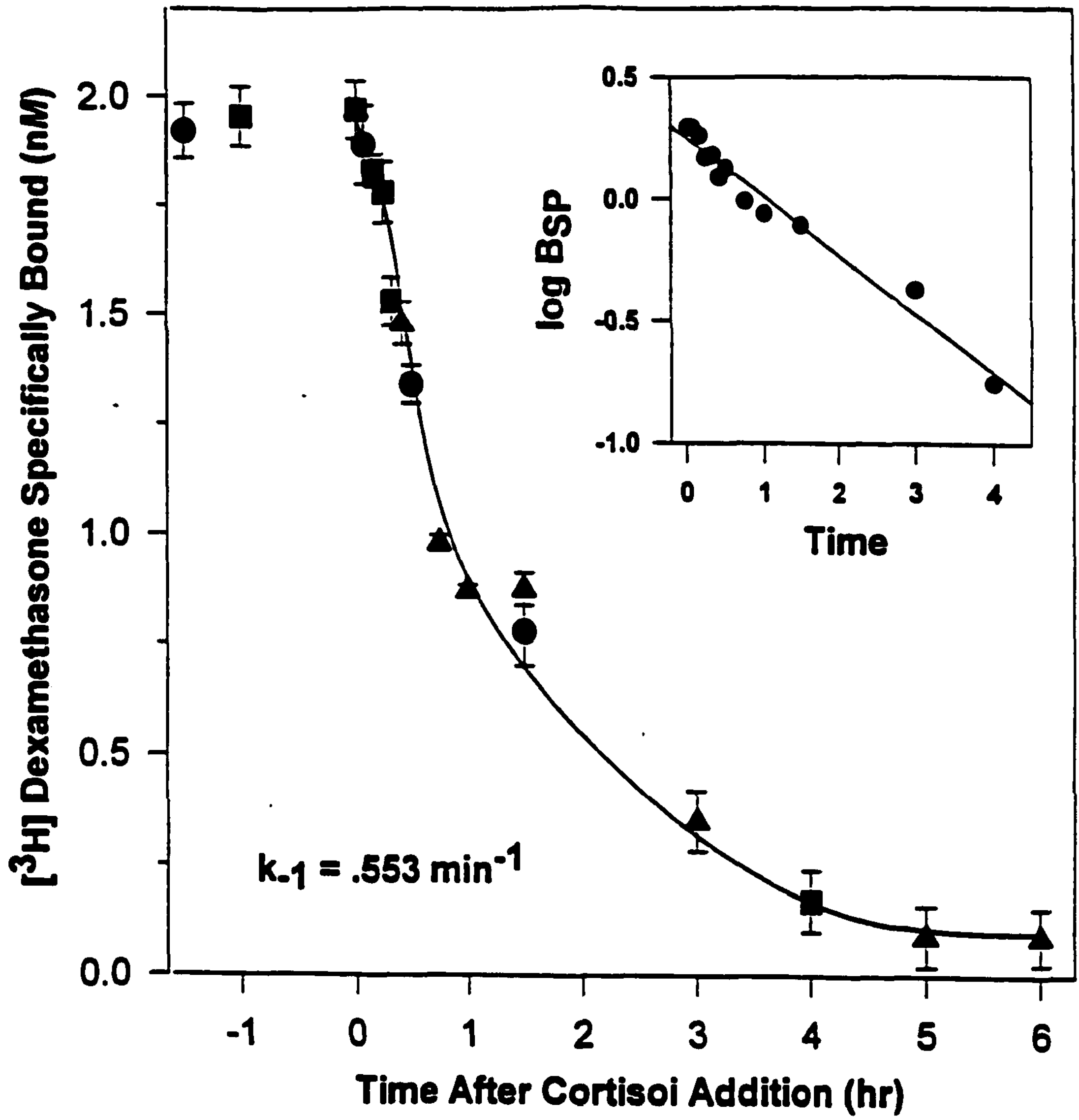

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FIG. 3. Dissociation of specifically bound (B_{SP}) [^{3}H]dexamethasone (n*M*) from hypothalamus cytosol preparations of juvenile rainbow trout. B_{SP} is the difference between binding in the absence (total binding; B_O) and presence (nonspecific binding; NSB) of 1000 molar excess cortisol. Dissociation was initiated by the addition of a 5000 molar excess cortisol and incubated up to 5 hr. Values are means (n= 4, ± SEM) from triplicate experiments (●, ■, ▲). Inset: Log B_{SP} as a function of time. Relationship (r^2 =0.97) was used to determine log B_{SP} = -0.24 (time) + 0.25 which was used to determine the first order dissociation rate constant (k_{-1}) of 0.553 min^{-1} (Bennett and Yamamura, 1985).

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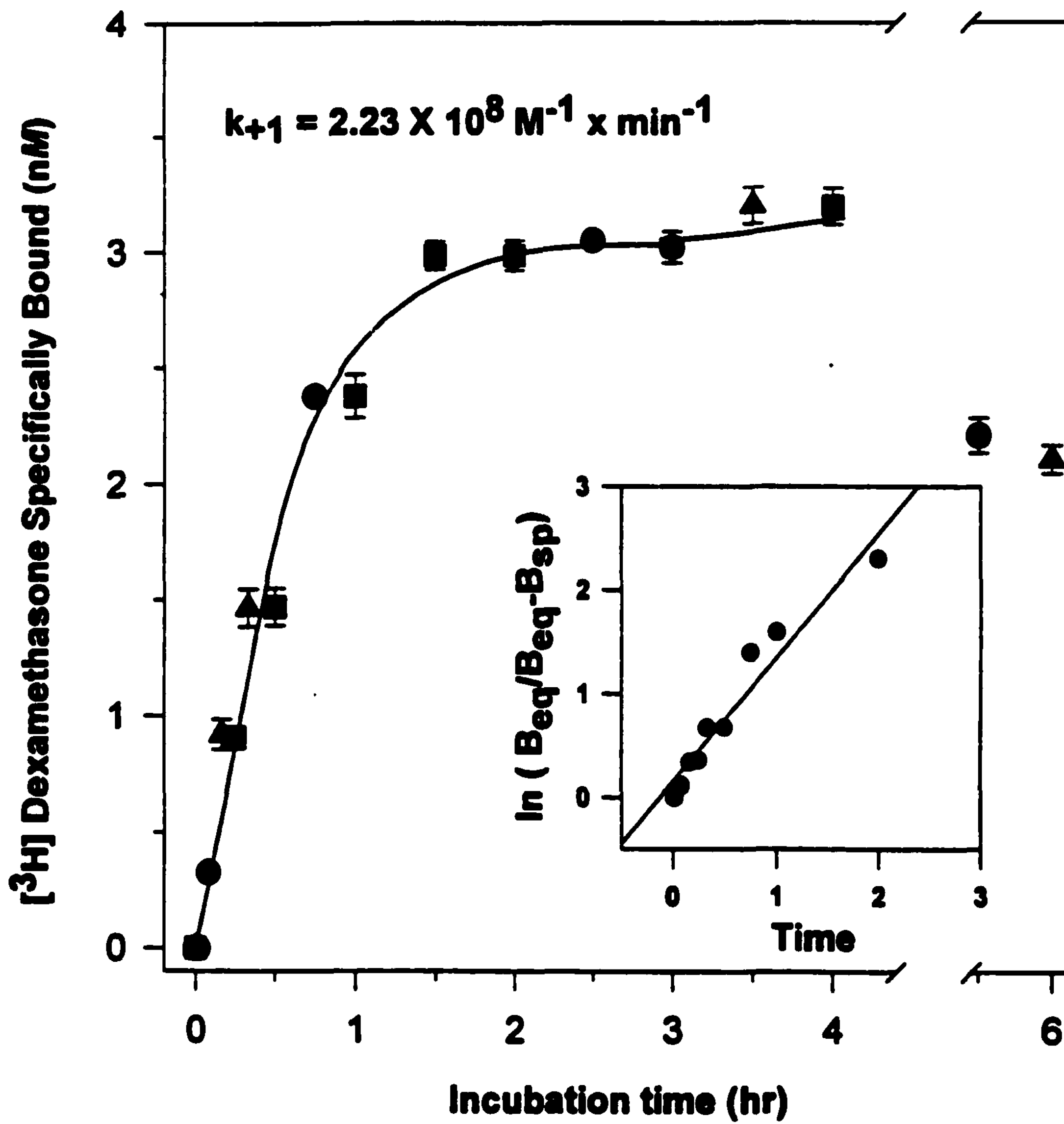

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FIG. 4. Specific binding (B_{SP}) of [^{3}H]dexamethasone (0.06-53.99 n*M*) to hypothalamus cytosol preparations of rainbow trout as a function of [^{3}H]dexamethasone (n*M*). B_{SP} is the difference between binding in the absence (total binding; B_O) and presence (nonspecific binding; NSB) of 1000 molar excess cortisol. Values are means (n= 4, ± SEM) from three independent experiments (●, ■, ▲). Inset: Scatchard analysis was used to determine K_d = 1.22 ± 0.20 n*M* and B_{MAX} = 296 ± 64.90 fmol x mg^{-1} protein (r^2=0.96).

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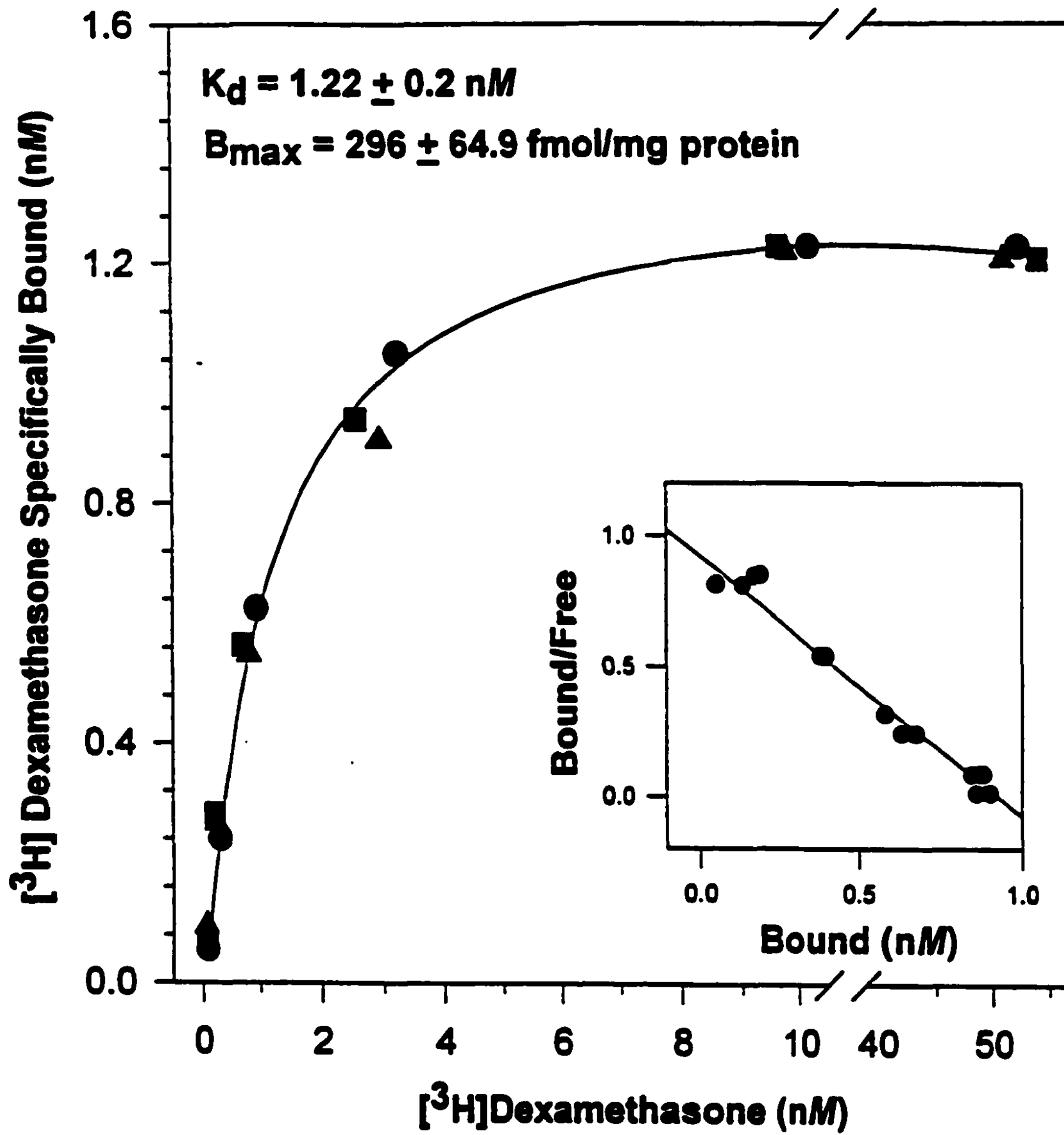

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FIG. 5. Displacement analysis of specific [^{3}H]dexamethasone binding (B_{SP}) to cytosol preparations of rainbow trout hypothalamus. Cytosol was incubated with [^{3}H]dexamethasone in the absence (total binding; B_O) or presence (nonspecific binding; NSB) of a 1000 molar excess of radioinert cortisol. B_{SP} is the difference between B_O and NSB. Percent of maximal binding is the difference between B_O and NSB for each competitor divided by B_{SP} (in the presence of 10μ*M* cortisol). Graphs display competitive inhibition of specific [^{3}H]dexamethasone binding by natural glucocorticoids and dexamethasone (A), synthetic glucocorticoids (B), and structurally related steroids (C). Values are means (n= 4, ± SEM) from three independent experiments.

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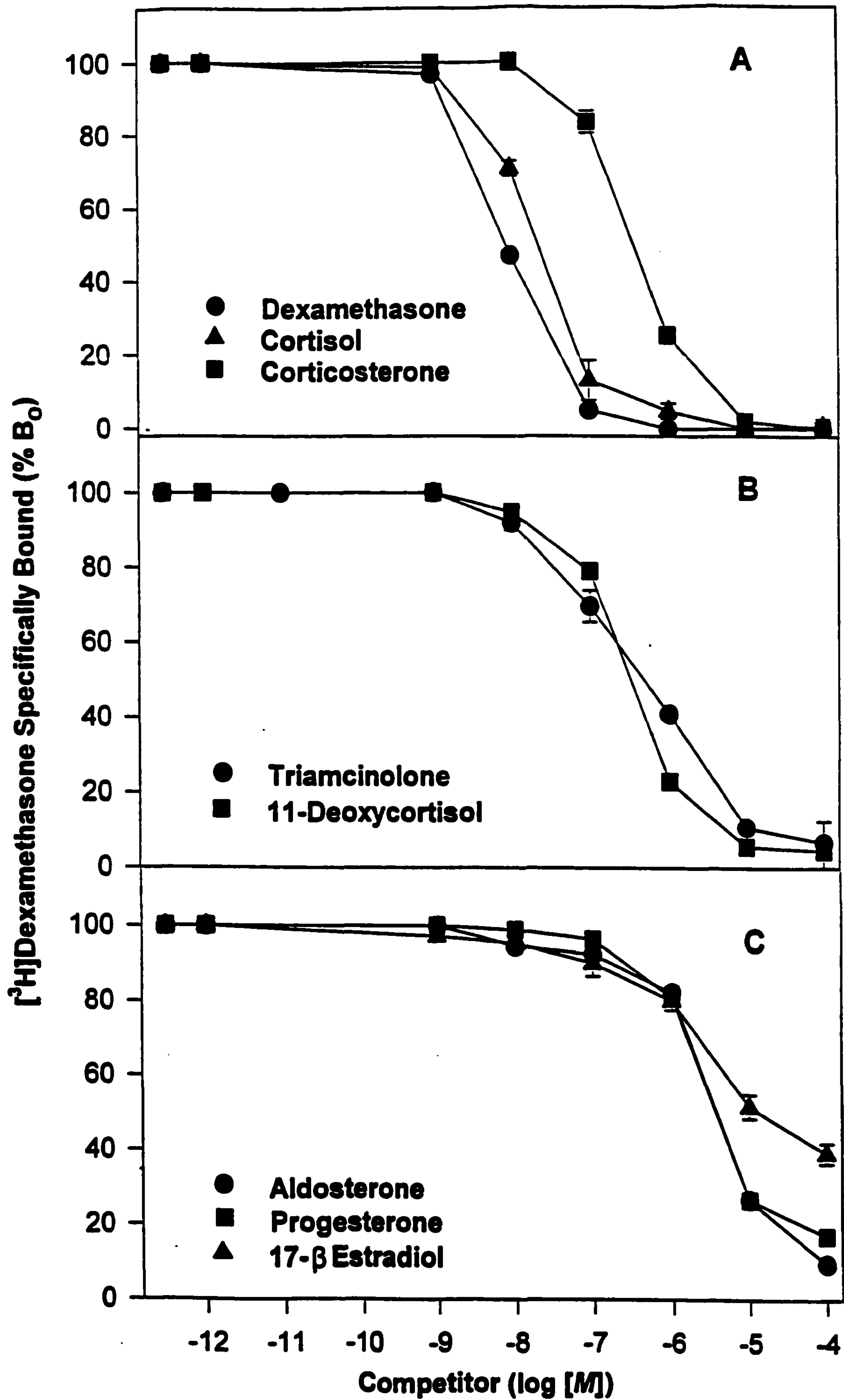

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TABLE 1

IC_{50} and K_d Values for Glucocorticoid and Nonglucocorticoid Competitors of [^{3}H]Dexamethasone Binding in the Hypothalamus of the Rainbow Trout, *Oncorhynchus mykiss*

Competitor	IC_{50} [n*M*]	K_d [n*M*]
Dexamethasone	10.7 ± 2.1	0.15
Cortisol	30.7 ± 8.2	0.46
Corticosterone	417 ± 14	1.90
Triamcinolone	659 ± 109	4.01
11-Deoxycortisol	623 ± 96	5.10
Aldosterone	> 1000	38.76
Progesterone	> 1000	>500
17β-Estradiol	> 5000	>5000

Note: Values are means (n= 4; ± SEM)from triplicate experiments. IC_{50} values were determined from linear regression of percent specific binding of [^{3}H]dexamethasone as a function of the log of the competitor concentration. K_d values were determined from Scatchard analysis (Scatchard, 1949) of maximally bound [^{3}H]dexamethasone displaced by competitor.

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CHAPTER 3

Allison, C.M. and Omeljaniuk, R.J. (1998). Binding characteristics of [^{3}H]17β-estradiol in the hypothalamus of the juvenile rainbow trout, *Oncorhynchus mykiss*.

Steroids (Submitted)

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ABSTRACT

Gonadal steroids in the salmonid brain, acting through cellular receptors, may be responsible for the modulation of neuronal activity and organization of reproductive behaviours. In this investigation, we report our findings on the use of [^{3}H]17β-Estradiol (E_2) to identify intracellular estrogen receptors (ERs) in the hypothalamus of juvenile rainbow trout, *Oncorhynchus mykiss*.

Specific binding (B_{SP}) (total binding - nonspecific binding) of [^{3}H]E_2 was tissue dependent between 0.5 and 2.25 hypothalamus equivalents for cytosol and nuclear extract preparations; cytosol and nuclear extract protein contents were 5.4 ± 0.8 and 0.51 ± 0.13 mg/hypothalamus, respectively. B_{SP} in cytosol fractions increased with time and reached maximum levels (4.18 n*M*) at 2.5 hrs incubation; by contrast, B_{SP} in nuclear extract increased with time to achieve maximum levels (3.9 n*M*) by 2 hrs incubation. The association rate constants (k_{+1}) for cytosol and nuclear extract preparations were $1.10 \pm 0.02 \times 10^8$ $M^{-1} \times min^{-1}$ and $1.27 \pm 0.04 \times 10^8$ $M^{-1} \times min^{-1}$, respectively. Equilibrium bound B_{SP} dissociated from cytosol preparations with a half life ($t_{1/2}$) of 42 min and a dissociation rate constant (k_{-1}) of 1.01 ± 0.03 min^{-1}; in contrast, B_{SP} dissociated from nuclear extract preparations with a $t_{1/2}$= 45 min and k_{-1} = 0.92 ± 0.01 min^{-1}. B_{SP} was saturable in both cytosol and nuclear extract preparations with calculated equilibrium dissociation constants (K_d) of 1.46 ± 0.1 n*M* and 2.37 ± 0.2 n*M*, respectively, and maximum number of binding sites (B_{MAX}) of 50.85 ± 3.2 fmol/mg protein and 61.74 ± 2.65 fmol/mg protein, respectively. In both preparations, B_{SP} was differentially displaced by estrogen-congeners and, to a lesser extent, by testosterone. Glucocorticoid analogues and nonsteroidal compounds displaced B_{SP} nonspecifically while the phytoestrogen, β-sitosterol, was completely ineffective. The rank order of potency for

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displacement of B_{SP} was E_2 > estrone > estriol > 17α-ethynyl estradiol > testosterone >> progesterone = tamoxifen >> cortisol > dexamethasone >>> β-sitosterol. These properties of specifically bound $[^3H]E_2$ suggest the presence of an ER in the hypothalamus of juvenile rainbow trout comparable with ERs identified in salmonid liver.

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INTRODUCTION

In bony fish (teleosts) 17β-estradiol (E_2) is the major circulating estrogen. E_2 participates in regulating varied functions including gonadal recrudescence (Hoar, 1969; Fostier *et al.*, 1983) and the production of yolk protien (vitellogenin) by the liver (Arcandhoy and Benson, 1998; Rinchard *et al.*, 1998). Synthesis and release of E_2 from ovarian follicles during the reproductive cycle are coordinated through the hypothalamic-pituitary-gonadal (HPG) axis (Fostier *et al.*, 1983; Peter, 1983; Kah *et al.*, 1997) which modulates rhythmic changes in E_2 secretion. In salmonids such as the rainbow trout (*Oncorhynchus mykiss*), gonadotropin-releasing hormone (GnRH) is synthesized in hypothalamic nuclei and applied directly to pituitary cells by direct synaptoid contact; GnRH directly stimulates release of the gonadotropic hormones, GtH I (in juvenile fish) and GtH II (in sexually mature fish) (Suzuki *et al.*, 1988; Weil and Marcuzzi, 1990; Breton *et al.*, 1998; Saligaut *et al.*, 1998). GtH II stimulates synthesis and release of the E_2 from ovarian follicle cells prior to ovulation. During the onset of ovulation, when spawning activities begin, there is rapid decline in E_2 secretion coincident with a rise in plasma GtH I levels (Scott and Sumpter, 1983). Fine tuning of E_2 secretion is thought to be governed by selective modulation of the HPG axis on E_2 negative feedback at the hypothalamus:pituitary complex through intracellular estrogen receptors (ERs) (Kah *et al.*, 1997).

ER mRNA has been found in close proximity to GnRH cell bodies in the rainbow trout hypothalamus (Salbert *et al.*, 1991; Anglade *et al.*, 1994). This evidence for ERs suggests a mechanism by which E_2 can modulate the release of gonadotropic hormones from a higher level within the HPG axis. The impact of E_2 on the feedback loop of the vertebrate HPG axis is illustrated by its influence on hypothalamic activity. In the rat brain, for example, the

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administration of E_2 suppresses GnRH synthesis (Rosie *et al.*, 1990; Llyod *et al.*, 1994); by contrast, E_2 increases GnRH-induced GtH II output from the pituitary of rainbow trout (Breton and Sambroni, 1996).

Although ERs at the level of the hypothalamus in teleosts may be important for regulating sexual behaviour and reproductive function, direct evidence is not yet available for the presence, distribution, and description of structural requirements for binding of ERs in specific areas such as the hypothalamus. We address this issue in this report of the existence and pharmacological characteristics of specific $[^3H]E_2$ binding sites in the hypothalamus of juvenile (pre-smolt) rainbow trout. This project was part of our ongoing research program investigating neuromodulatory actions of estrogen and other classes of steroids in the teleost brain.

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METHODS AND MATERIALS

Animals. Fingerling rainbow trout (Rainbow Springs Hatchery, Thamesford, Ontario, Canada) were raised to juvenile stage (possessing distinct parr marks) and maintained at the Lakehead University Aquatic Animal Research Facility in aquaria supplied with flow-through, dechlorinated water at simulated ambient temperature (5 to 16° C, annual range) and photo period (8 to 16 hr, annual range). Fish were fed daily *ad libitum* with commercial trout pellets (Martin trout pellets, Thunder Bay Co-Op). Prior to handling, fish were anaesthetized with tricaine methanesulfonate (MS 222, 0.05g/litre; Syndel Laboratories, Vancouver, BC) and killed by spinal transection posterior to the medulla oblongata.

Tissue Preparation. Hypothalami were excised and immediately transferred to a polystyrene tube, immersed and stored in liquid nitrogen, and assayed within 24hr. The hypothalamus was defined as the region ventral to the thalamus and posterior to the telencephalon. Dissection commenced at the optic tract and extended posteriorly to the nucleus diffusus lobi inferioris (Peter and Gill, 1975). This receptor assay was based on a modification of the methods of Campbell *et al.* (1994). All steps were carried out at 0-4°C unless stated otherwise. Pooled hypothalami were thawed in 100µl/hypothalamus of ice-cold TEDMS buffer (10 m*M* Tris; 1 m*M* EDTA; 1 m*M* dithiothreitol; 20 m*M* sodium molybdate; 250 m*M* sucrose; 10% (v/v) glycerol; pH 7.4), homogenized on ice using 10 strokes of a motor-driven Teflon-glass homogenizer (0.125 mm clearance) and centrifuged at 1500g for 20 min to obtain a crude nuclear pellet and cytosol (supernatant). The supernatant was decanted into an equal volume of dextran-coated activated charcoal suspension (DCC; 0.125% dextran, 1.25% charcoal in TEDMS, Sigma Chemicals, St. Louis, MO), incubated for 45 min with frequent vortexing to remove endogenous steroids, then

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centrifuged for 1 hr at 40,000g , and the cytosol preparation (supernatant) used directly in the receptor assay.

Protocol to obtain the nuclear extract was based on a modification of the methods of Lazier *et al.* (1985), Pottinger and Pickering (1990), and Campbell *et al.* (1994). The crude nuclear pellet was washed three times with an original volume of TEDS buffer (10 m*M* Tris; 1 m*M* EDTA; 1 m*M* dithiothreitol; 250 m*M* sucrose; 10% (v/v) glycerol; pH 7.4), resuspended in an original volume of TEDK buffer (0.7 *M* KCl in TEDS buffer), then frequently vortexed during a 1 hr incubation. After centrifugation (1500g x 10 min) the supernatant (nuclear extract) was decanted into a polystyrene tube, and frozen in liquid nitrogen until assayed. Prior to use, the extract was thawed and incubated (45 min with frequent vortexing) in an equal volume of DCC suspension to remove endogenous steroids. The mixture was then centrifuged (1500g x 10 min), the supernatant collected and used in the receptor assay. Liver was used as a positive control tissue to validate the protocol (data not shown). 100µL aliquot of liver tissue (1g/9 volumes TEDS buffer) was incubated for 6 hours with 100µL [^{3}H]E_2 (4.0 n*M*) in the presence (nonspecific binding) and absence (total binding) of 1000-fold molar excess E_2. Specific binding of [^{3}H]E_2 in liver cytosol was 3.1 n*M*.

Radioreceptor Assay.. A 100µl aliquot of hypothalamic extract was incubated under various experimental paradigms with 100µl of [2,4,6,7-^{3}H]17β-Estradiol ([^{3}H]E_2) (84.1 Ci/mmol; NEN-Dupont, Boston, MA) in the absence (total binding, B_O) or presence (nonspecific binding, NSB) of 100µl of a 1000-fold molar excess of radioinert E_2 (Sigma Chemicals, St. Louis, MO) in a final volume of 300µl in 12x75 mm glass tubes. Incubations were terminated by the addition of 300µl of DCC to remove free [^{3}H]E_2 from the supernatant , tubes were vortexed, incubated for 10 min,

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then centrifuged (1500g x 10 min). A 400μl aliquot of the supernatant containing bound [^{3}H]E_2 was placed in a 6 ml scintillation vial in combination with 4 ml of liquid scintillation cocktail (Ready Safe, Beckman Instruments Inc., Fullerton, CA), mixed, and allowed to incubate overnight in the dark. Sample radioactivity was determined by liquid scintillation spectroscopy using a Beckman LS-6500 liquid scintillation spectrometer (50% counting efficiency).

Specific Investigations

[^{3}H]E_2 binding to hypothalamic equivalents of cytosol and nuclear extract preparations. Various dilutions of cytosol and nuclear extract were incubated in quadruplicate, in three independent experiments, for 3 hr with [^{3}H]E_2 in the absence (B_O) or presence (NSB) of 1000-fold molar excess E_2 prior to termination. One hypothalamus-equivalent per tube was used in subsequent experiments since that amount of tissue routinely provided a substantial signal in the linear range of tissue dilutions.

Association of [^{3}H]E_2 to hypothalamus cytosol and nuclear extract preparations. In three independent experiments, trout cytosol and nuclear extract preparations were incubated in quadruplicate with [^{3}H]E_2 in the absence (B_O) or presence (NSB) of 1000-fold molar excess E_2 for various intervals prior to termination.

Dissociation of [^{3}H]E_2 from hypothalamus cytosol and nuclear extract preparations. Cytosol and nuclear extract were incubated in quadruplicate with [^{3}H]E_2 in the absence (B_O) or presence (NSB) of 1000-fold molar excess E_2 for 3 hr to establish equilibrium binding conditions. Thereafter all tubes received a 5000-fold molar excess of radioinert E_2, tubes were then vortexed and incubated for various intervals prior to termination. Independent experiments were performed in triplicate.

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Saturation analysis of [$^3H]E_2$ binding to hypothalamus cytosol and nuclear extract preparations. In three independent experiments, cytosol and nuclear extract were incubated in quadruplicate for 3 hr with various concentrations of [^{3}H]E_2 in the absence (B_O) or presence (NSB) of 1000-fold molar excess E_2 prior to termination.

Competitive displacement analysis of [$^3H]E_2$ binding to hypothalamus cytosol and nuclear extract preparations. In triplicate experiments, cytosol and nuclear extract were incubated with [^{3}H]E_2 in quadruplicate in the absence (B_O) or presence (NSB) of competitors at various concentrations. Reaction mixtures were incubated for 3 hr prior to termination. Competitors, (Sigma Chemicals, St. Louis, MO), represented major steroid groups such as estrogen (17β-estradiol, estriol, estrone, and 17α -ethynyl estradiol), androgens (testosterone; Steraloids Inc., Wilton, NH), glucocorticoids (cortisol, dexamethasone and triamcinolone), progestins (progesterone), and β-sitosterol, a common plant sterol. Tamoxifen, a nonsteroidal antiestrogen used in breast cancer therapy, was also utilized.

Protein Assay. Protein content was determined by the method of Bradford (1976) using Coomassie Brilliant Blue G-250 as the dye reagent (Sigma Chemicals, St. Louis, MO) with bovine serum albumin (Sigma Chemicals, St. Louis, MO) as the protein standard.

Data Analysis. Specific binding (B_{sp}) was calculated as the difference between total (B_O) and nonspecific (NSB) binding. Means were calculated from three independent replications of each experiment where each experiment consisted of 4 samples taken from a pool of hypothalami; the standard error of the mean , B_{sp} (SEM), was calculated as $(\sigma^2_{Bsp} + \sigma^2_{NSB})^{1/2}$ (Hulme and Birdsall, 1992).

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First order transformations of kinetic data were performed according to Bennett and Yamamura (1985) to determine association and dissociation rate constants, k_{+1} and k_{-1}, respectively. k_{-1} was calculated from the linear regression analysis of log B_{SP} versus time where k_{-1} = slope of the line. k_{obs} was calculated from the plot of $\ln(B_{eq}/B_{eq}-B_{sp})$ versus time where B_{eq} is the level of binding at equilibrium and the k_{obs} = slope of the line. k_{+1} was estimated from $(k_{obs} - k_{-1})/[L]$; where k_{obs} = the observed rate of association and [L] = the concentration of free $[^3H]E_2$.

Equilibrium binding data from saturation experiments were subjected to Scatchard analysis (Scatchard, 1949) to estimate the equilibrium dissociation constant (K_d, nM) and the maximum number of binding sites (B_{MAX}, fmol/mg protein). By comparison, equilibrium binding data from competitive displacement experiments were analysed according to the methods of Hulme and Birdsall (1992). Briefly, binding data were plotted as ln (P/1.0-P) as a function of competitor concentration (log, nM); P values are the decimal ratios of maximal B_{sp} (P = 1.0) (Hulme and Birdsall, 1992). The half-maximal inhibitory concentration (IC_{50}) values for each competitor was estimated from linear regression analysis of plotted data; the plot of. IC_{50} = -B/M. Where Y = MX + B. In addition, K_d and B_{MAX} values for each competitor were estimated from Scatchard analysis of binding data (Scatchard, 1949). Statistical comparison of binding parameters, such as IC_{50}, between cytosol and nuclear preparations were made on the basis of Mann-Whitney *U*-tests using Statistical Package for Social Sciences (SPSS) (SPSS Inc., Chicago, IL). P-values < 0.05 were considered significant.

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RESULTS

$[^3H]E_2$ binding to hypothalamic equivalents of cytosol and nuclear extract preparations. B_{SP} of $[^3H]E_2$ to cytosol and nuclear extract preparations increased with the amount of tissue present (FIG. 1). For both preparations B_{SP} increased linearly between 0.5 to 2.25 hypothalamus-equivalents per tube with a signal range of 0.86 to 3.4 n*M* for cytosol and 0.90 to 3.62 n*M* for nuclear extract. One hypothalamus-equivalent per tube was used in subsequent experiments. The protein content of a 1-hypothalamus-equivalent was 5.4 ± 0.8 mg (n=9, ± SEM) for cytosol and 0.51 ± 0.13 mg (n=9, ± SEM) for nuclear extract.

Association of $[^3H]E_2$ to hypothalamus cytosol and nuclear extract preparations. B_{SP} of $[^3H]E_2$ to cytosol and nuclear extract preparations increased slowly with time with the first significant increase in B_{SP} after 45 min and 30 min incubation, respectively (FIG. 2). Equilibrium binding levels of 4.18n*M* for cytosol and 3.9 n*M* for nuclear extract were achieved after 2.5 hr and 2.0 hr incubation, respectively and remained stable for at least 3 hours thereafter. For both preparations, pooled data from three independent experiments were used to estimate $k_{+1} = 1.10 \pm 0.02 \times 10^8 M^{-1} \times min^{-1}$ (n= 3, ± SEM) for cytosol and $k_{+1} = 1.27 \pm 0.04 \times 10^8 M^{-1} \times min^{-1}$ (n= 3, ± SEM) for nuclear extract preparations, according to the method of Bennett and Yamamura (1985)

Dissociation of $[^3H]E_2$ from hypothalamus cytosol and nuclear extract preparations. Equilibrium bound $[^3H]E_2$ rapidly dissociated from cytosol and nuclear extract preparations after the addition of a 5000-fold molar excess of radioinert E_2. Complete dissociation of specifically bound $[^3H]E_2$ was achieved within 2 hr for each tissue preparation; addition of excess E_2 did not cause further dissociation of $[^3H]E_2$ in NSB tubes. For each tissue preparation, pooled data from three independent experiments were used to estimate (Bennett and Yamamura, 1985) the

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dissociation rate constant (k_{-1}) of 1.01 ± 0.03 min^{-1} (n= 3, ± SEM) for cytosol and k_1 = 0.92 ± 0.01 min^{-1} (n= 3, ± SEM) for nuclear extract. Cytosol preparations had a kinetically derived dissociation constant (k_{-1}/k_{+1}) of 9.18 x 10^{-9} *M* with an estimated half life ($t_{1/2}$) of 42 min; by comparison, the nuclear extract preparation had a kinetically derived dissociation constant of 7.24 x 10^{-9} *M* with a slightly longer half life ($t_{1/2}$) of 45 min (FIG. 3).

Saturation analysis of [^{3}H]E_2 binding to hypothalamus cytosol and nuclear extract preparations. Specific binding of [^{3}H]E_2 to cytosol and nuclear extract increased to maximum levels with [^{3}H]E_2 concentrations (FIG. 4). Saturable [^{3}H]E_2 specifically bound to cytosol (3.69 n*M*) was lower than saturable [^{3}H]E_2 specifically bound to nuclear extract (5.13 n*M*.). Scatchard analyses of cytosol and nuclear extract data were linear relationships (r^2 = 0.91 and r^2 = 0.94, respectively) suggesting a single class of binding sites. Both cytosol and nuclear extract preparations possessed high-affinity (K_d = 1.46 ± 0.1 n*M* and K_d = 2.37 ± 0.2 n*M*, respectively; n=3, ± SEM) and low-capacity binding sites (B_{MAX} = 50.85 ± 3.20 fmol/mg protein and B_{MAX} = 61.74 ± 2.65 fmol/mg protein, respectively; n=3, ± SEM) (FIG. 4).

Competitive Displacement Analysis of [^{3}H]E_2 binding to hypothalamus cytosol and nuclear extract preparations. For cytosol and nuclear extract preparations, the natural estrogen were the most effective competitors with sigmoidal displacement curves indicative of first-order, receptor:ligand interactions. The endogenous hormone E_2 (IC_{50} = 13.5 ± 0.4 n*M* for cytosol and IC_{50} = 9.1 ± 0.2 n*M* for nuclear extract; n=3, ± SEM) and its metabolite, estrone (IC_{50} = 28.1 n*M* ± 1.1 for cytosol and IC_{50} = 30.2 ± 0.6 n*M* for nuclear extract; n=3, ± SEM) were the strongest competitors for [^{3}H]E_2 binding in the hypothalamus. Estriol, a metabolite of estrone, was less effective (IC_{50}= 95.9 ± 3.4 n*M* for cytosol and IC_{50}= 102 ± 6.8 n*M* for nuclear extract; n=3, ±

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SEM) in its ability to displace specifically bound [^{3}H]E_2. 17α-ethynyl estradiol (a synthetic estrogen), and testosterone (progenitor of E_2) had IC_{50} values that were an order of magnitude higher than E_2 (Table 1). Progesterone, tamoxifen, cortisol, and dexamethasone non-specifically displaced [^{3}H]E_2, a suggested by their non-sigmoidal displacement of [^{3}H]E_2 (FIG 5-6). β-sitosterol did not displace specifically bound [^{3}H]E_2 in either cytosol or nuclear extract preparations of rainbow trout hypothalamus. Only E_2 showed a significant difference ($U = 0.0$, 4 df, $P = 0.0495$) in mean IC_{50} values between cytosol and nuclear extract preparations. The rank order of potency was E_2 > estrone > estriol > 17α-ethynyl estradiol > testosterone >> progesterone = tamoxifen >> cortisol > dexamethasone >>> β-sitosterol (FIG. 5,6).

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DISCUSSION

We present evidence for specific $[^3H]E_2$ bindings sites whose binding characteristics suggest the existence of specific ERs in the hypothalamus of juvenile rainbow trout. In both cytosol and nuclear extract preparations, $[^3H]E_2$ binding was dependent on the amount of tissue used, while association with its binding sites proceeded quickly, and was reversible. Both preparations were characterized by the presence of saturable, high-affinity ($K_d = 1.46 \pm 0.1$ nM, cytosol; $K_d = 2.37 \pm 0.2$ nM, nuclear extract), low capacity ($B_{MAX} = 50.85 \pm 3.2$ fmol/mg protein, cytosol; $B_{MAX} = 61.74 \pm 2.65$ fmol/mg protein, nuclear extracts) binding sites. Similar binding affinities have been observed for hepatic ERs in rainbow trout ($K_d = 2.2 \pm 0.5$ n*M*, cytosol; $K_d = 4.2 \pm 0.8$ n*M*, nuclear) (Pottinger and Pickering, 1990; Campbell *et al.*, 1994) and brown trout ($K_d = 2.9 \pm 0.3$ n*M*, cytosol; $K_d = 2.6 \pm 0.2$ n*M*, nuclear) (Pottinger, 1986).

The juvenile trout used in this assay display a level of binding sites similar to that of hepatic ERs in rainbow trout prior to ovulation ($B_{MAX} = 65 \pm 8.6$ fmol/mg protein) (Campbell *et al.*, 1994). The amount of E_2 secreted into plasma of immature fish is considerably reduced compared to levels once sexual maturation begins (Scott *et al.*, 1983). Throughout sexual development alterations in tissue sensitivity to E_2 can occur via changes in the number of ERs. However, such changes are influenced by the gender and degree of sexual development of the animal. To illustrate, sexually mature (pre-ovulatory) brown trout (*Salmo trutta* L.) ($B_{MAX} = 168 \pm 15$ fmol/mg protein) (Pottinger, 1986) and rainbow trout (*Oncorhynchus mykiss*) ($B_{MAX} = 137 \pm 13.9$ fmol/mg protein) are characterized as having higher concentrations of hepatic ERs compared to mature male brown trout ($B_{MAX} = 69 \pm 9.0$ fmol/mg protein) (Pottinger, 1986) and rainbow trout ($B_{MAX} = 37.2 \pm 2.6$ fmol/mg protein) (Campbell *et al.*, 1994).

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This putative ER in hypothalamus of juvenile rainbow trout is structurally selective for a variety of compounds with the highest binding affinities demonstrated for those compounds possessing an aromatic ring or oxygen substituent groups on carbon C-3 and C-17 (features normally associated with estrogen ligand-recognition) (Henzl, 1991). In this assay, the specificity of [^{3}H]E_2 binding in the trout was demonstrated for a variety of competitors (Table 1). The natural estrogen possess a high degree of structural similarity and readily displaced specifically bound [^{3}H]E_2 in both the cytosol and nuclear extract preparations. E_2, the primary follicular hormone, was the strongest competitor (IC_{50}= 13.5 ± 0.4 for cytosol and IC_{50}= 9.1 ± 0.2 for nuclear extract; n= 3, ± SEM). Estrone, produced from the oxidation of E_2 and present in plasma throughout the entire reproductive cycle (Hoar, 1969), was less effective (IC_{50}= 28.1 ± 1.1 for cytosol and IC_{50}= 30.2 ± 0.6, for nuclear extract; n= 3, ± SEM) than E_2. The least competitive natural estrogen was estriol (IC_{50}= 95.9 ± 3.4 for cytosol and IC_{50}= 102 ± 4.7 for nuclear extract; n= 3, ± SEM). It is a metabolite of estrone which is predominant in plasma during spawning (Hoar, 1969).

17α-ethynyl estradiol (EE), a synthetic estrogen, and testosterone were similar in their ability to displace specifically bound [^{3}H]E_2 with both having IC_{50} values an order of magnitude lower than E_2. Both compounds possess differences in fundamental structures which may contribute to their reduced efficacy compared to the natural estrogen. Specifically, EE has an ethyl group added in the α-position on C-17, whereas testosterone lacks an aromatic ring structure and has a methyl group on C-10. Progesterone, cortisol, and dexamethasone were not effective competitors, possibly due to the absence of the aromatic ring structure and the presence of additional substituents groups on C-17. It is important to note that estrogen was not an

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effective competitor of corticosteroid binding in the trout hypothalamus (Allison and Omeljaniuk, 1998). Although the level of hepatic ERs in salmonids has been shown to be sensitive to changes in the circulating levels of cortisol (Pottinger and Pickering, 1990), a stress-related hormone that also undergoes enhanced secretion during spawning (Pickering *et al.*, 1987; Scott *et al.*, 1983), there is no apparent cross reactivity of these hormones between corticosteroid and E_2 receptors. Tamoxifen had to be used at concentrations of 2.5 x 10^5 n*M* (cytosol) and 1.3 x 10^5 n*M* (nuclear extract) to achieve 50 percent displacement of specifically bound [^{3}H]E_2. Similar results were obtained using tamoxifen to displace [^{3}H]E_2 from hepatic ERs of Atlantic salmon, *Salmo salar* (Lazier *et al.*, 1985) and the spotted sea trout (Smith and Thomas, 1990). The reduced efficacy of this compound as a competitor may be due its nonsteroidal structure. However, β-sitosterol, a common plant sterol found in pulp mill effluent (Strömberg *et al.*, 1996), was unable to displace specifically bound [^{3}H]E_2 within the limits of this assay ($\leq$ 1 x 10^4 n*M*). Despite its structural similarities to E_2, β-sitosterol lacks two structures common to estrogen (Henzl, 1991). The absence of an aromatic ring A and the replacement of the oxygen substituent group on the C-17 may be responsible for its ineffectiveness as a competitor. In general, the relative potencies of these compounds as competitors of [^{3}H]E_2 binding is comparable to that found in the spotted seatrout (Smith *et al.*, 1990), brown trout (Pottinger, 1986), and Atlantic salmon (Lazier *et al.*, 1985).

The binding of [^{3}H]E_2 in the hypothalamus of juvenile rainbow trout is consistent with the current model for steroid hormone receptors. According to this model, ERs are considered to be of nuclear origin in both its active (hormone bound) and inactivated states (free of hormone). This idea has been supported by the identification of nuclear ERs in the rat pituitary in the absence of

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bound ligand (Welshons *et al.*, 1984). Activated ERs possess a high affinity for DNA and can only be extracted with the use of high ionic strength buffers. The unoccupied ERs, however, have a lower affinity for DNA and can be extracted with low ionic strength buffers, accounting for their appearance in cytosol fractions. The presence of specific E_2 binding sites in both cellular compartments is supported by evidence for ERs in teleosts (Lazier *et al.*, 1985; Pottinger and Pickering, 1990; Smith and Thomas, 1990; Smith and Thomas, 1991; Campbell *et al.*, 1994; Todo *et al.*, 1995).

Until recently, only a single type of ER was known to mediate genomic activity associated with E_2 binding in vertebrate tissues. Since its discovery in rat prostate (Kuiper *et al.*, 1996), the novel ER subtype (ERβ) has been identified throughout the brain with the highest expression of ERβ mRNA in the hypothalamus of the rat (Shughrue and Merchenthaler, 1996; Li *et al.*, 1997; Nilsson and Gustafsson, 1997) and mouse (Couse *et al.*, 1997), but absent in the pituitary of both animals (Couse *et al.*, 1997; Nilsson and Gustafsson, 1997). While both ERα and ERβ subtypes are present in both sexes, ERβ:ERα mRNA expression in the mouse hypothalamus is approximately 0.5:1 in females and 3:1 in males (Couse *et al.*, 1997). Recently, ER mRNA has been localized in the rainbow trout brain (Begay *et al.*, 1994), including the hypothalamus (Salbert *et al.*, 1991). While it has been suggested that ERs at in this region may be important for regulating teleost sexual behaviour and reproductive function, the existence of ER subtypes and their role in HPG axis activity have yet to be elucidated.

In conclusion, we have presented evidence for intracellular ERs in the hypothalamus that may provide a locus for E_2 modulation of the HPG axis. To our knowledge, this is the first attempt to characterize $[^3H]E_2$ binding-sites in the salmonid hypothalamus.

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FIG. 1. Specific binding (B_{SP}) of [^{3}H]E_2 to hypothalamus cytosol and nuclear extract preparations of juvenile rainbow trout. B_{SP} is the difference between binding in the absence (total binding; B_O) and presence (nonspecific binding; NSB) of 1000 molar excess E_2. A linear relationship between B_{SP} and tissue content ([^{3}H]E_2 specifically bound = 1.65 (tissue content) + 0.02, r^2 = 0.99, cytosol; [^{3}H]E_2 specifically bound = 1.52 (tissue content) + 0.06, r^2 = 0.99, nuclear) was observed between 0.5 and 2.25 hypothalamus equivalents. Values are means (n= 4, ± SEM) from three independent experiments (●, ■, ▲).

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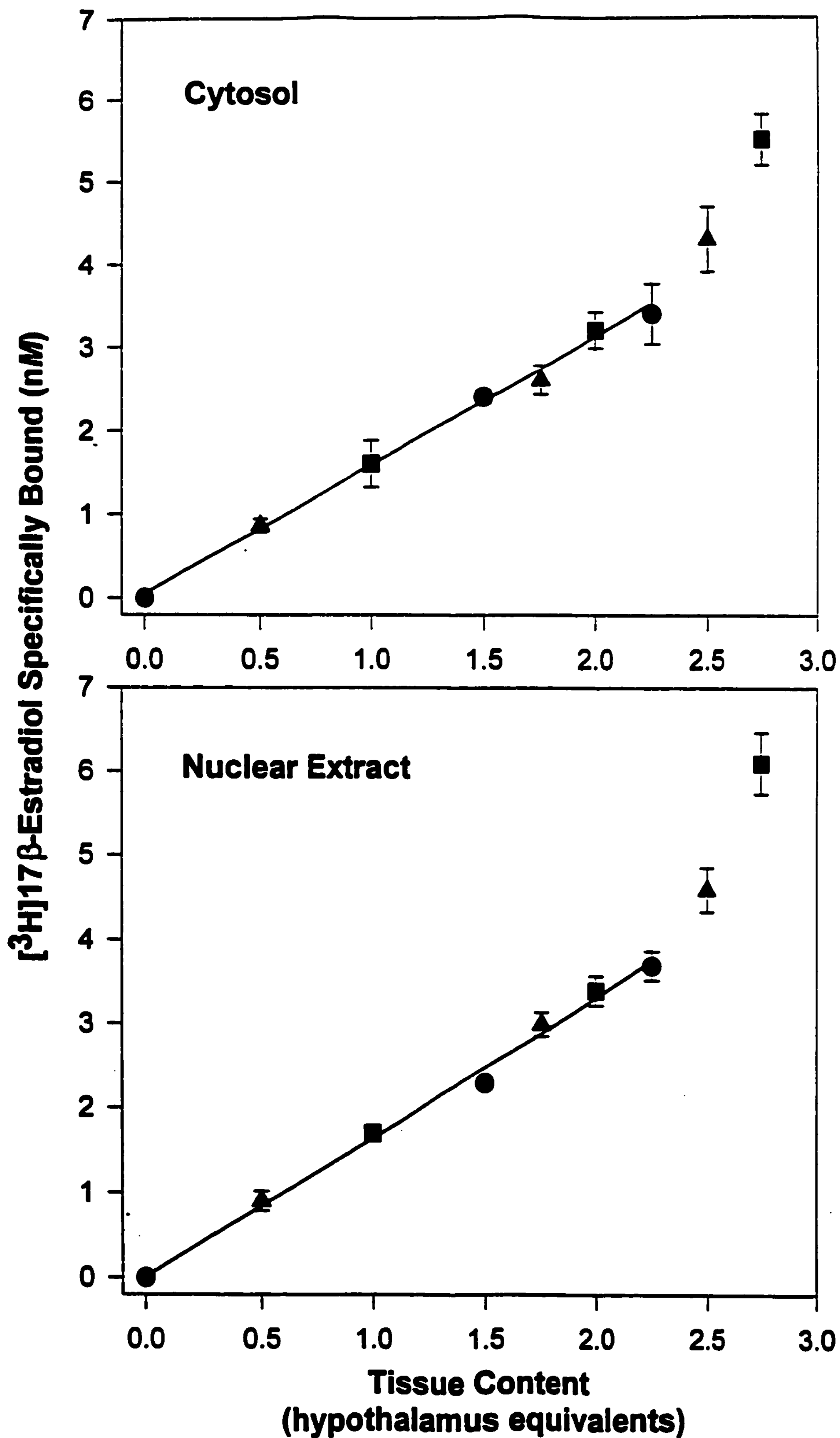

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FIG. 2. Specific binding (B_{SP}) of [^{3}H]E_2 to hypothalamus cytosol and nuclear extract preparations of juvenile rainbow trout as a function of time. Specific binding (B_{SP}) is the difference between binding in the absence (total binding) and presence (nonspecific binding) of 1000 molar excess E_2. Values are means (n= 4, ± SEM) from three independent experiments (●, ■, ▲). Inset A: ln (B_{eq}/B_{eq}-B_{SP}) as a function of time. The linear relationship (r^2=0.97) was used to determine ln (B_{eq}/B_{eq}-B_{SP}) = 1.45 (time) - 0.51; association rate constant (k_{+1}) (Bennett and Yamamura, 1985) was 1.10 ± 0.02 x 10^8 M^{-1} x min^{-1} for cytosol. Inset B: ln (B_{eq}/B_{eq}-B_{SP}) as a function of time. The linear relationship (r^2=0.93) was used to determine ln (B_{eq}/B_{eq}-B_{SP}) = 1.43 (time) - 0.17; k_{+1} was calculated as 1.27 ± 0.04 x 10^8 M^{-1} x min^{-1} for nuclear extract. B_{eq} is the equilibrium level of B_{SP}.

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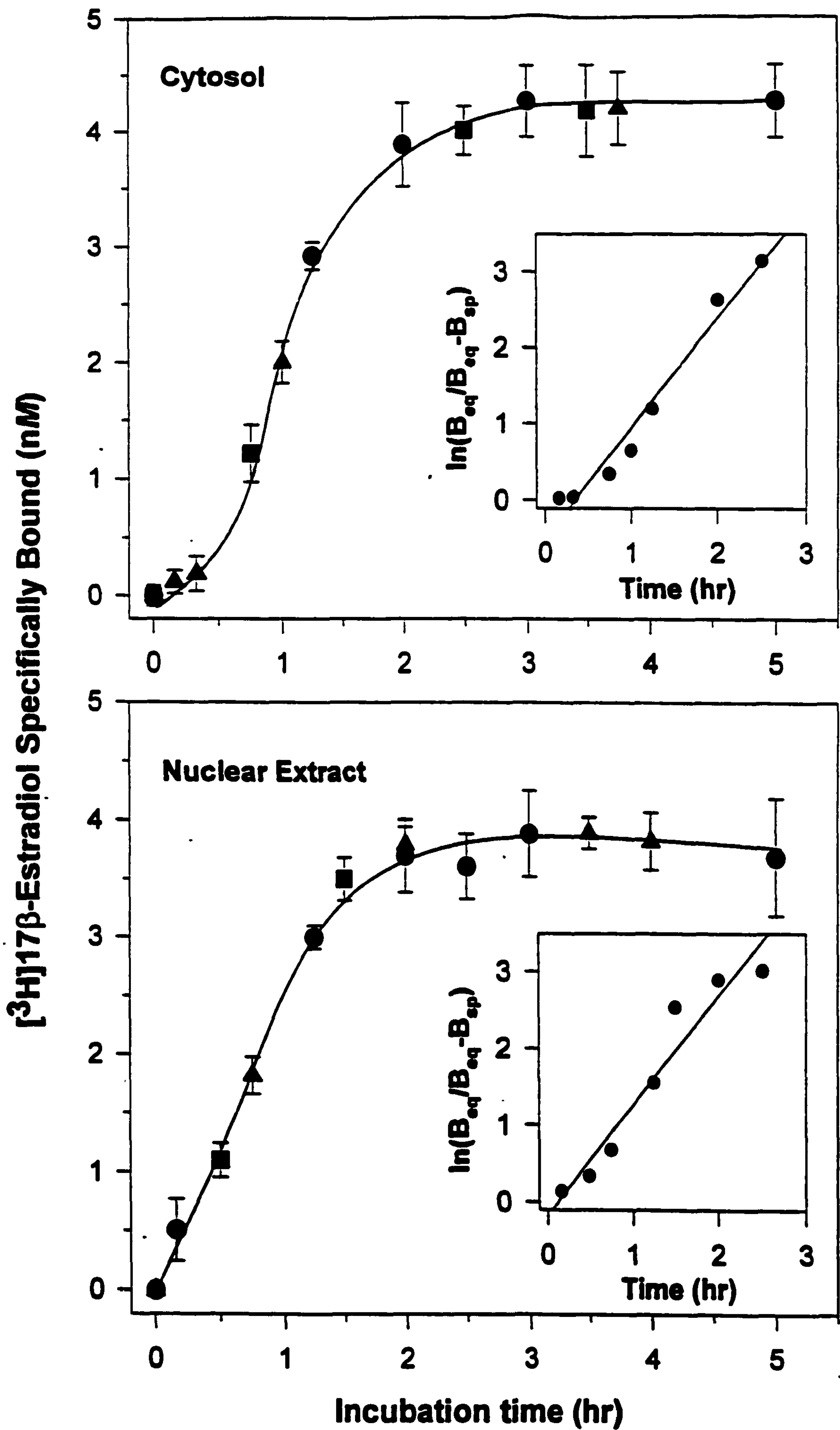

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FIG. 3. Dissociation of specifically bound (B_{sp}) [^{3}H]E_2 from hypothalamus cytosol and nuclear extract preparations of juvenile rainbow trout. B_{sp} is the difference between binding in the absence (total binding) and presence (nonspecific binding) of 1000 molar excess E_2. Dissociation was initiated by the addition of a 5000 molar excess E_2 and incubated up to 5 hr. Values are means (n= 4, ± SEM) from triplicate experiments (●, ■, ▲). Inset A: log B_{sp} as a function of time. The linear relationship (r^2 =0.95) was used to determine log B_{sp} = -0.44 (time) + 0.33; the first order dissociation rate constant (k_{-1}) (Bennett and Yamamura, 1985) was 1.01± 0.03 min^{-1}. Inset B: log B_{sp} as a function of time. The linear relationship (r^2 =0.93) was used to determine log B_{sp} = -0.40 (time) + 0.21; k_{-1} was 0.92 ± 0.01min^{-1}.

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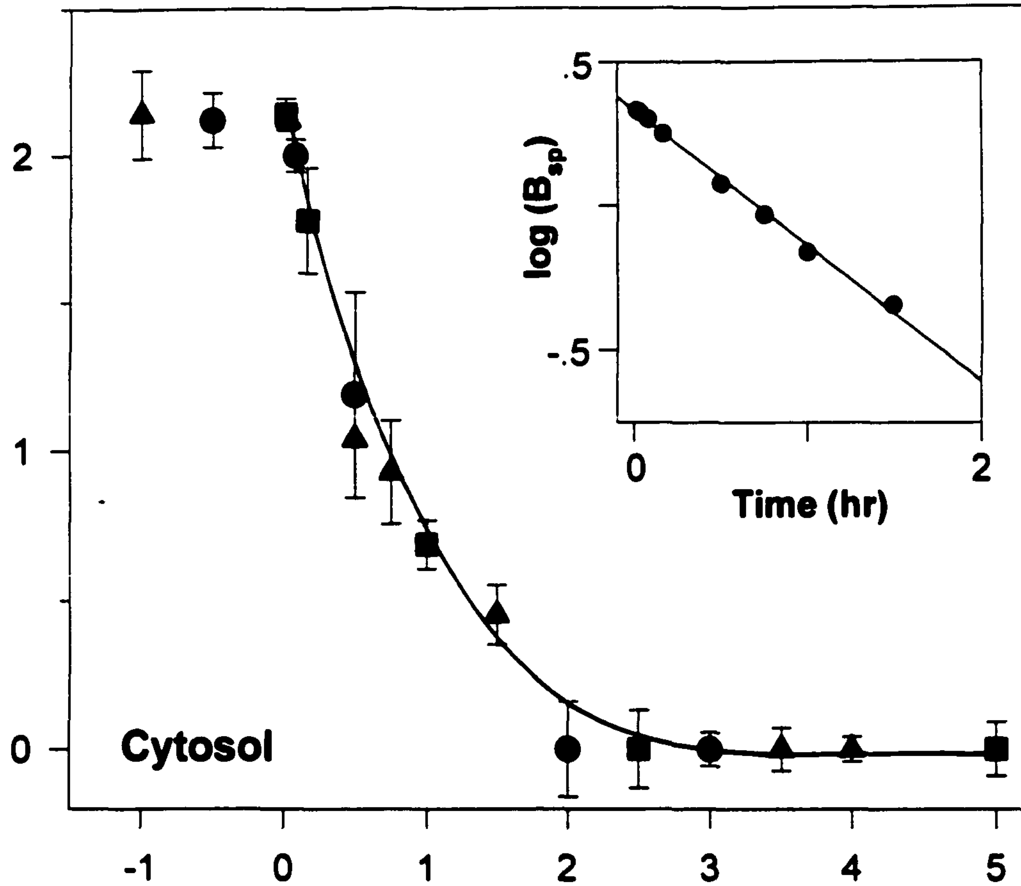

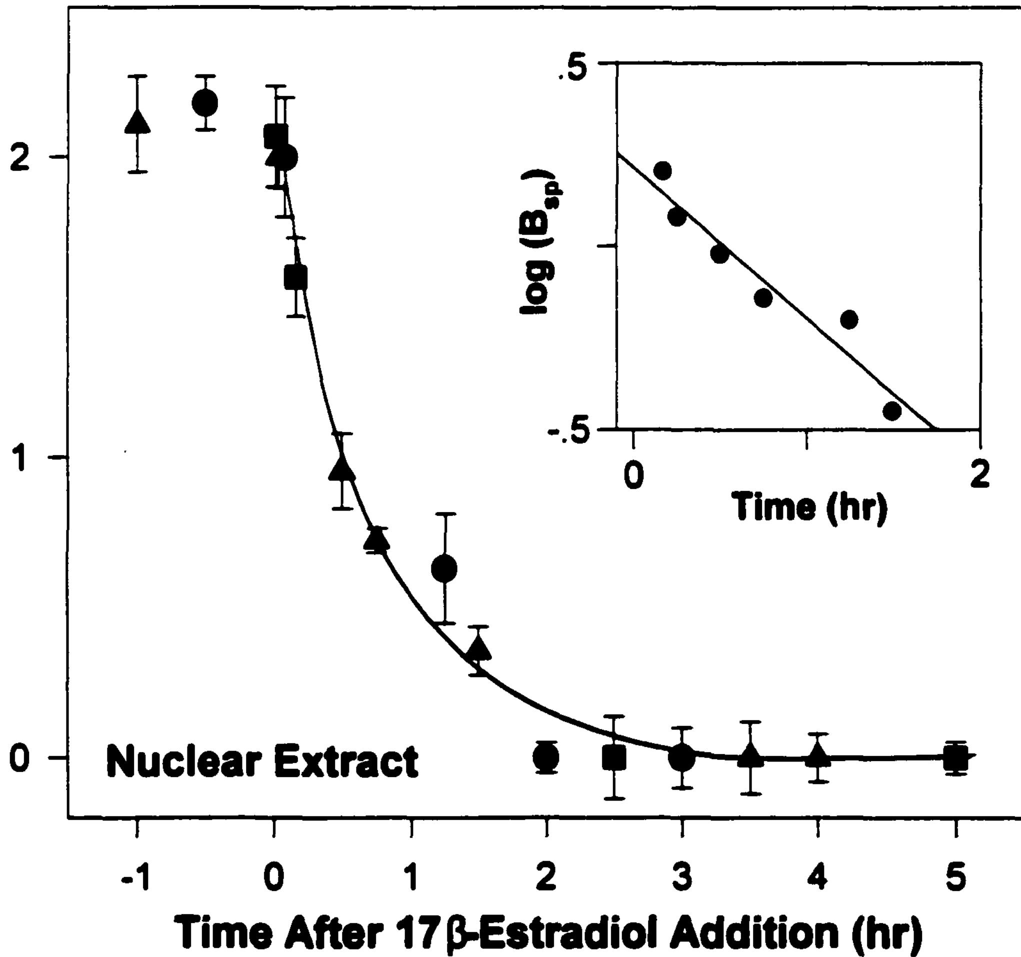

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FIG. 4. Total (B_O, ●), nonspecific (NSB, ■) and specific binding (B_{SP}, ▲) of $[^3H]E_2$ to hypothalamus cytosol and nuclear extract preparations of rainbow trout as a function of $[^3H]E_2$ (0.08-32.41 n*M*). B_{SP} is the difference between binding in the absence (B_O) and presence (NSB) of 1000 molar excess E_2. Values are means (n= 4, ± SEM) from three independent experiments. Scatchard analysis (Scatchard, 1949) of data was used to determine cytosol K_d = 1.46 ± 0.20 n*M* and B_{MAX} = 50.85 ± 3.20 fmol x mg^{-1} protein (n= 3, ± SEM, r^2=0.91) and nuclear extract K_d = 2.37 ± 0.20 n*M* and B_{MAX} = 61.74 ± 2.65 fmol x mg^{-1} protein (n= 3, ± SEM, r^2=0.94).

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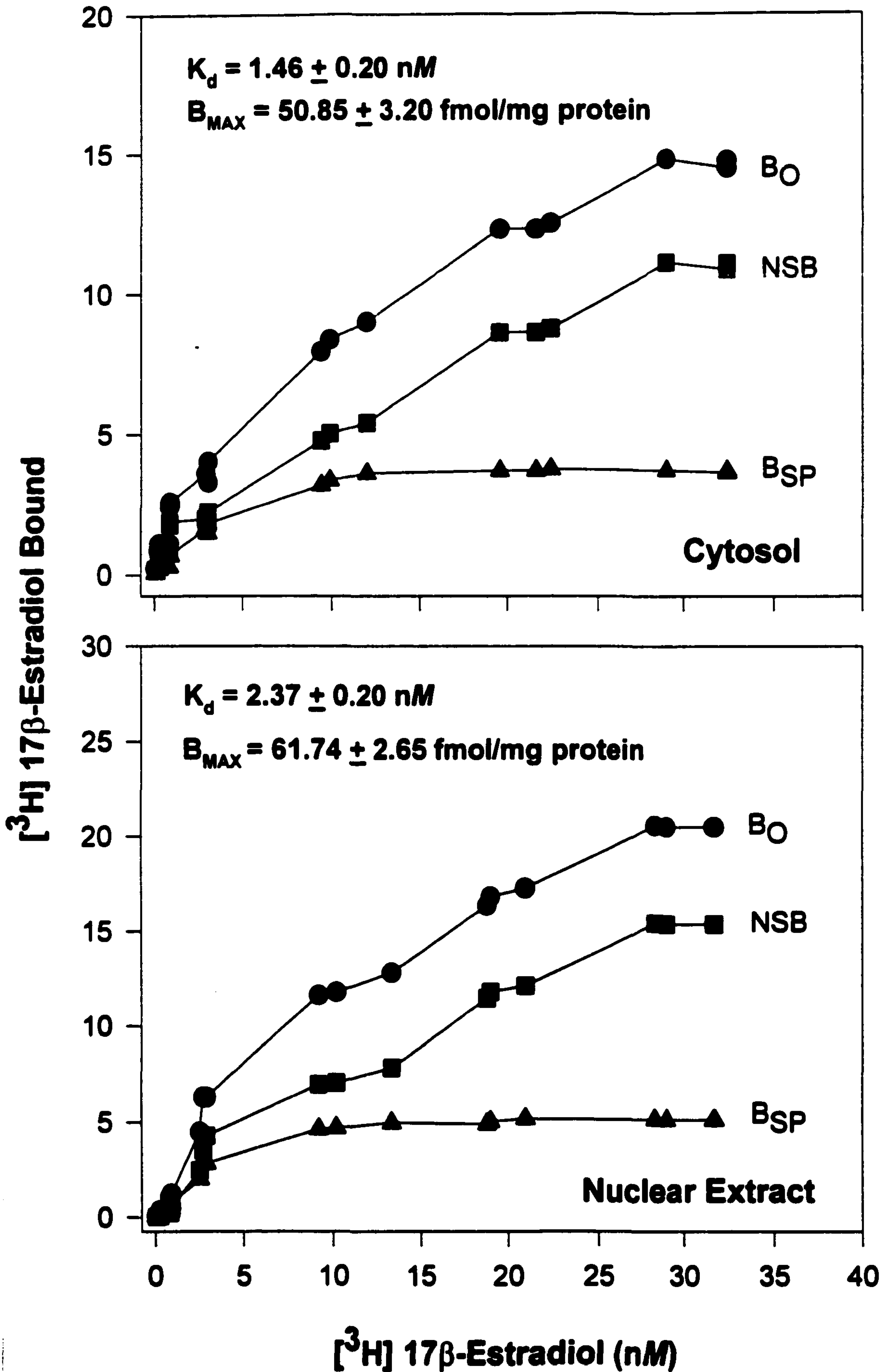

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FIG. 5. Displacement analysis of specific [^{3}H]E_2 binding (B_{SP}) to cytosol preparations of rainbow trout hypothalamus. Cytosol was incubated with [^{3}H]E_2 in the absence (total binding; B_O) or presence (nonspecific binding; NSB) of a 1000 molar excess of radioinert E_2. B_{SP} is the difference between B_O and NSB. Percent of specific binding is the difference between B_O and NSB for each competitor divided by B_{SP} (in the presence of 10μ*M* E_2). Graphs display competitive inhibition of specific [^{3}H]E_2 binding by estrogen (A), androgens and glucocorticoids (B), and related compounds (C). Values are means (n= 4, ± SEM) from three independent experiments. Plots are best fit lines.

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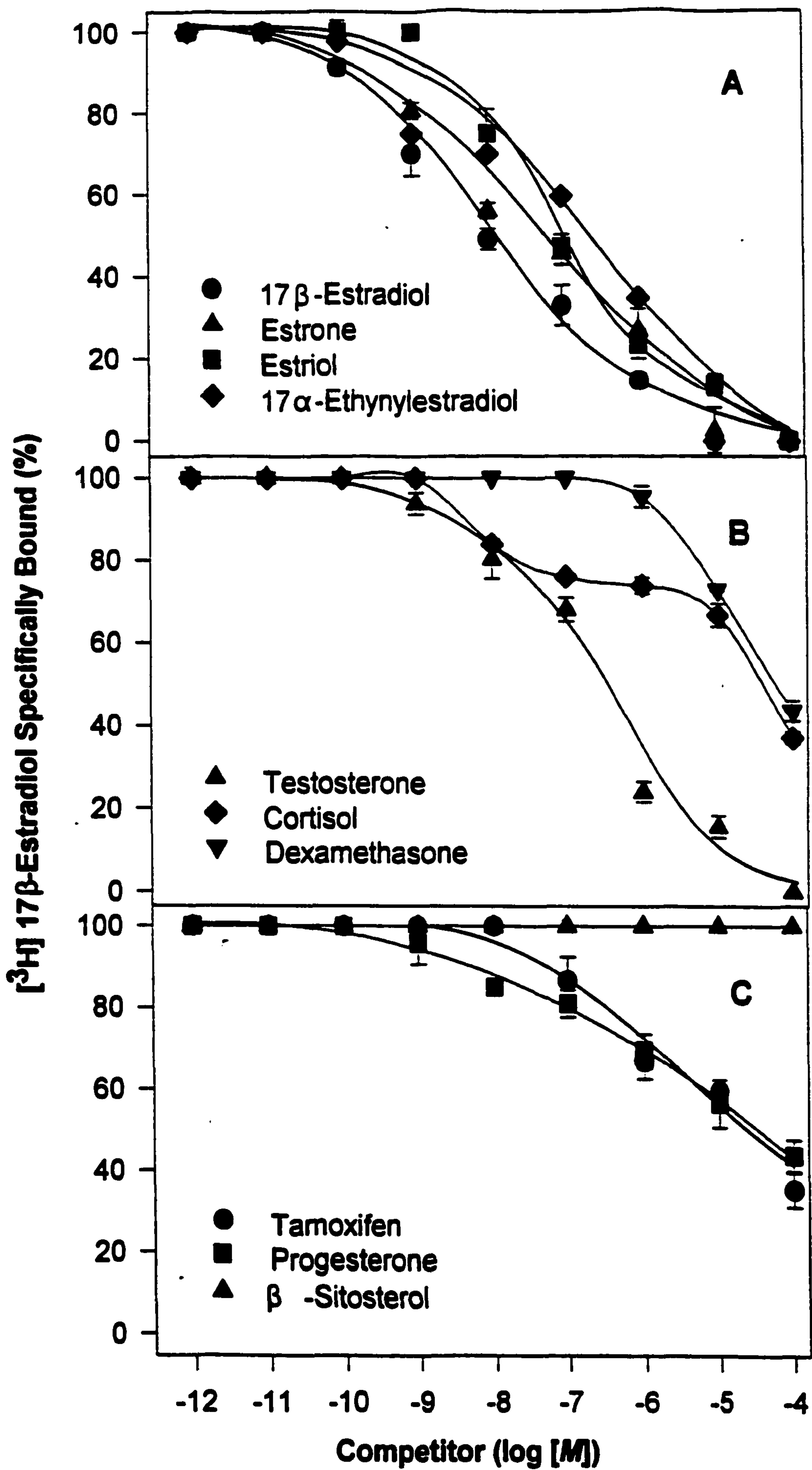

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FIG. 6. Displacement analysis of specific [^{3}H]E_2 (B_{SP}) to nuclear extract preparations of rainbow trout hypothalamus. Nuclear extract was incubated with [^{3}H]E_2 in the absence (total binding; B_O) or presence (nonspecific binding; NSB) of a 1000 molar excess of radioinert E_2. B_{SP} is the difference between B_O and NSB. Percent of specific binding is the difference between B_O and NSB for each competitor divided by B_{SP} (in the presence of 10μ*M* E_2). Graphs display competitive inhibition of specific [^{3}H]E_2 binding by estrogen (A), androgens and glucocorticoids (B), and related compounds (C). Values are means (n= 4, ± SEM) from three independent experiments. Plots are best fit lines.

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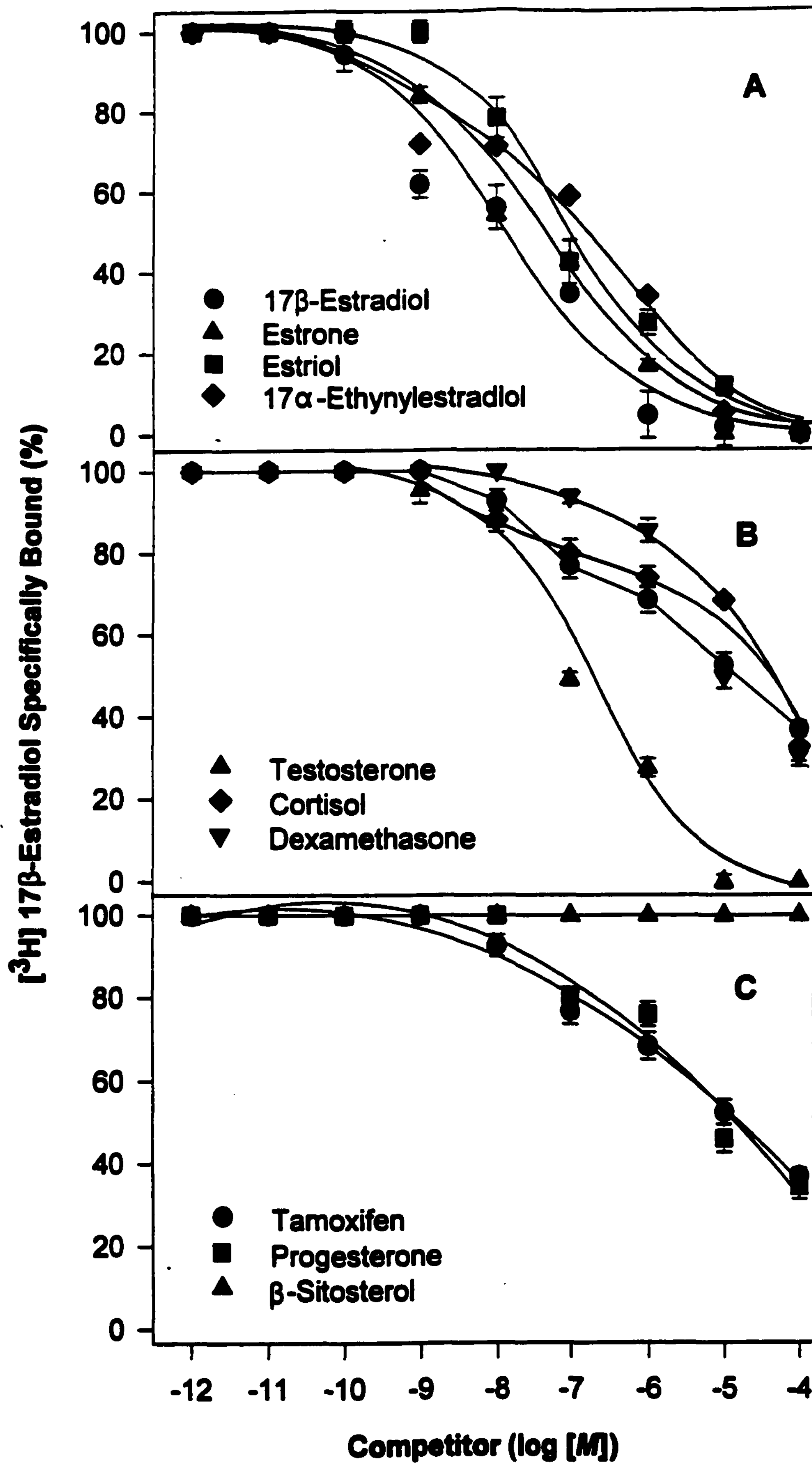

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TABLE 1

IC_{50} and K_d Values for Estrogenic and Nonestrogenic Competitors of [^{3}H]17β-Estradiol Binding in the Hypothalamus of the Rainbow Trout, *Oncorhynchus mykiss*

	IC_{50} [n*M*]		K_d [n*M*]	
Competitor	**Cytosol**	**Nuclear**	**Cytosol**	**Nuclear**
17β-estradiol	13.5 ± 0.4[a]	9.1 ± 0.2[b]	2.7 ± 0.5	3.0 ± 0.1
Estrone	28.1 ± 1.1[c]	30.2 ± 0.6[c]	23.2 ± 4.8	19.6 ± 1.8
Estriol	95.9 ± 3.4[d]	102 ± 4.7[d]	114 ± 11.5	93.4 ± 6.1
17α-ethynyl estradiol	179 ± 7.4[e]	168 ± 6.8[e]	158 ± 17.6	141 ± 3.6
Testosterone	230 ± 13.9[f]	224 ± 11.1[f]	207 ± 21.5	201 ± 17.9
Progesterone	> 1000	> 1000	> 1000	> 1000
Tamoxifen	> 1000	> 1000	> 1000	> 1000
Cortisol	> 3000	> 3000	> 3000	> 3000
Dexamethasone	> 2000	> 2000	> 2000	> 2000

Note: Values are means (n= 4; ± SEM) from triplicate experiments. IC_{50} values were determined from linear regression of percent specific binding of [^{3}H]17β-estradiol (E_2) as a function of the log of the competitor concentration. K_d values were determined from Scatchard analysis (Scatchard, 1949) of maximally bound [^{3}H]E_2 displaced by competitor. Mean values superscripted with the same letter are not significantly different based on Mann-Whitney *U* tests at $P > 0.05$.

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CHAPTER 4

General Discussion

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My findings describing the specific binding properties of [^{3}H]DEX (Chapter 2) and [^{3}H]17β-E_2 (Chapter 3) in the trout hypothalamus provide strong evidence for the existence and pharmacological properties of corticosteroid and estrogen receptors, respectively, in the trout brain. Moreover, these findings predicate a central neuromodulatory role for these hormones in modulating the HPI and HPG axes.

It has been well documented that two types of corticosteroid receptors are present in the mammalian system: type I receptors which bind mineralocorticoids with a high affinity and GCs with a low affinity, and type II receptors which preferentially bind GCs (DeKloet *et al.*, 1993). In teleosts , however, only one type of corticosteroid receptor has been detected. A single species of GR mRNA has been localized throughout the forebrain of rainbow trout showing greatest density within CRH-releasing neurons of the hypothalamus (Teitsma *et al.*, 1997).

As previously indicated in Chapter 2 of this thesis, putative GR levels ($B_{MAX} = 296 \pm 64$ fmol/mg protein) and binding affinity ($K_d = 1.22 \pm 0.20$ n*M*) are in accordance with that found in other salmonid tissues such as gill epithelia (Sandor *et al.*, 1984; Chakraborti *et al.*, 1987; Maule and Schreck, 1991; McLeese *et al.*, 1994), liver (Chakraborti and Weisbart, 1987; Pottinger, 1990; Lee *et al.*, 1992; Pottinger *et al.*, 1994) and whole brain preparations (Lee *et al.*, 1992; Knoebl *et al.*, 1996) under physiological conditions. [We can speculate that fish used in the present study had not been recently exposed to stressors associated with elevated plasma cortisol levels since there does not appear to be significant downregulation of GRs. It has been demonstrated that GR levels decline by as much as 60% within 24 hours after the onset of a stress event (Pottinger, 1990). In the absence of literature pertaining to stress-induced and seasonal changes in hypothalamic GR activity in teleosts it is difficult to comment specifically at this time

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as to how the results of this study compare to basal GR activity in this region.] In addition, my findings suggest the existence of a receptor-mediated feedback mechanism at the level of the hypothalamus that may allow these fish to modulate corticosteroid secretion in a manner similar to that described for the mammalian HPA axis.

The data presented in Chapter 3 of this thesis provide the first characterization of a putative ER in the hypothalamus of the juvenile rainbow trout. Binding parameters for cytosol (B_{MAX} = 50.85 ± 3.20 fmol/mg protein, K_d = 1.46 ± 0.10 n*M*) and nuclear extracts (B_{MAX} = 61.74 ± 2.65 fmol/mg protein, K_d = 2.37 ± 0.20 n*M*) are consistent with findings on hepatic ERs in salmonids prior to sexual maturation (Pottinger, 1986; Pottinger and Pickering, 1990; Smith and Thomas, 1991; Campbell *et al.*, 1994). As with GRs, seasonal studies on salmonid ER binding characteristics at the level of the hypothalamus are lacking, making it difficult to speculate on how plasma E_2 levels relate to ER activity at this level in the HPI axis. In general, sexually mature male brown trout (Pottinger, 1986) and rainbow trout (Campbell *et al.*, 1994) have hepatic ER levels (B_{MAX} = 168 and 137 fmol/mg protein, respectively) that are an order of magnitude higher than those observed in mature female fish (B_{MAX} = 69 and 37 fmol/mg protein, respectively) which are exposed to elevated plasma E_2 levels. The juvenile rainbow trout utilized in this study had not completely undergone smoltification (parr marks still evident), thus low hypothalamic ER levels described in this thesis may indicate a limited sensitivity of their HPG axis in keeping with a low E_2 exposure of sexually immature salmonids. As in the GR study, my findings on the specific $[^3H]E_2$ binding at the level of the hypothalamus in the trout suggest the existence of a mechanism for modulating HPG axis activity via a receptor-mediated feedback loop similar to that found in mammals. However, low levels of ERs demonstrated in the immature fish used in this study may

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be indicating reduced HPG axis activity (i.e. low sensitivity to E_2) in juvenile fish due to the absence of elevated sex steroid secretion at this stage in their life history, rather a downregulation of higher ER levels found in mature fish.

Both [^{3}H]DEX and [^{3}H]E_2 binding sites in the hypothalamus demonstrate a high degree of ligand specificity favoring structurally related steroidal compounds. Preference for ligand binding in this putative GR appears to be most sensitive to substitutions of hydroxyl groups on C11 and C21 and of methyl groups on C18. Whereas selectivity of ligand binding by the putative ER depends on the presence of an aromatic ring A structure, the presence of an hydroxyl group on C3 and C17, and a hydrogen substituent on C11. β-sitosterol, a common nonsteroidal plant sterol found in pulp mill effluent, is thought to have estrogenic activity in fish, however, it was not an effective competitor for [^{3}H]E_2 binding in the hypothalamus of the juvenile rainbow trout. In humans, β-sitosterol accumulates in all tissues except the brain (Lutjohann and Vonbergmann, 1997) and contributes to a reduction in the synthesis of cholesterol (Lutjohann and Vonbergmann, 1997; Honda *et al.*, 1998), a precursor for steroid synthesis. Similar decreases in cholesterol availability in response to β-sitosterol exposure may also account for reduced plasma sex steroid levels in both male and female goldfish (Maclatchy and Van der Kraak, 1995; Maclatchy *et al.*, 1997). While it appears that β-sitosterol could have a significant effect on teleost reproductive function, it may be limited in its impact on the HPG axis, since it has the capacity to affect the synthesis of gonadal hormones (Maclatchy and Van der Kraak, 1995), but does not interact at the level of the pituitary (Maclatchy and Van der Kraak, 1995) or the hypothalamus (Allison and Omeljaniuk, 1998). Despite considerable evidence for variations in GR activity throughout the reproductive development of salmonid fishes, E_2 was not an effective competitor of [^{3}H]DEX

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binding in the hypothalamus. Its influence on the HPA axis may not occur at this organizational level in juvenile rainbow trout.

GC IMPACT ON HPG AXIS AND ITS PRODUCTS

The HPA axis is an essential system that allows vertebrates to limit changes in their physiologic status during stressful events by providing a mechanism which can return the axis to its pre-stress state. However, the effects of stress can extend beyond the HPA axis activities often impacting on an animal's reproductive activities (Selye, 1950). To illustrate, female rats have a more robust HPA axis response to stress, yet HPA axis products can inhibit reproductive function in both sexes (Handa *et al.*, 1994).

As in mammals, various stressors impact on the reproductive activities of fish by influencing such aspects as the female:male sex ratio (van den Hurk and van Oort, 1985), the secretion of sex steroids (Pickering *et al.*, 1987), or by affecting the number and quality of gametes (Carragher *et al.*, 1989). In general, salmonid fishes display a sex-based difference in their sensitivity to cortisol exposure. For example, the administration of cortisol or cortisone, a major metabolite of cortisol, to rainbow trout fry (300 days post fertilization) has been shown to inhibit ovarian growth and promote the development of greater numbers of male fish (van den Hurk and van Oort, 1985). In addition, maturing male trout are characterized by having a plasma cortisol level half that of females after cortisol administration, while chronically elevated plasma cortisol coincides with smaller gonad size, lower plasma vitellogenin levels, and reduced pituitary GtH content in sexually maturing female brown trout and rainbow trout, but not males (Carragher *et al.*, 1989). It is also apparent that the stress response coincides with an altered release of both HPI and HPG axis products to modify the reproductive function of fish. Plasma cortisol

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elevations occurring as a result of acute (1 hour) handling stress in sexually mature brown trout was shown to coincide with enhanced plasma ACTH and GtH levels lasting for 4 hours, but lowered plasma testosterone (T) and 11-ketotestosterone (11-KT) levels for up to 24 hours (Pickering *et al.*, 1987). Similarly, dramatic reductions (~50% decrease) in plasma T and 11-KT levels have been observed during the period of elevated plasma cortisol associated with chronic stress in mature brown which failed to acclimate after a month of physical confinement (Pickering *et al.*, 1987) and in Atlantic salmon inhabiting acidic river systems (Freeman *et al.*, 1983).

Differences in cortisol sensitivity also exist at various stages of sexual maturity in salmonids where immature rainbow trout show an enhanced response to acute stressors than mature fish (Sumpter *et al.*, 1987) and where elevated plasma cortisol levels in sexually maturing rainbow trout inhibit E_2 secretion from ovarian follicles with more mature follicles showing the greatest sensitivity of E_2 suppression (~90% decrease) by cortisol (Carragher and Sumpter, 1990). The repercussions of stress on the reproductive function of fish can be seen in the sex-based and developmental differences in cortisol sensitivity that coincide with alterations of GR levels in the tissues involved. Pottinger and Pickering (1990) demonstrated cortisol's ability to alter hepatic ERs in juvenile rainbow trout where administration of cortisol resulted in a 35% and 29% decline in the number cytosolic and nuclear ERs, respectively, which persisted for 2 to 4 weeks in the absence of a concurrent change in plasma E_2 levels. Such persistently low ER levels demonstrated by the stressed juvenile trout may be due to a concurrent limited ER sensitivity during a period of reduced HPG axis activity when sex steroid secretion is low prior to the onset of sexual development in these fish.

E_2 IMPACT ON HPA/HPI AXIS AND ITS PRODUCTS

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It is well known that sex-based differences exist in the mammalian response to stress. Originally these differences were thought to exist only at the level of adrenal steroid synthesis and secretion (Kitay, 1963), but this has been extended to include gender-specific differences in neurendocrine activity. Specifically, an animal's estrogen status affects the HPA axis response to ACTH, corticosteroid, and GR-mediated functions (Burgess and Handa, 1992). For example, E_2 treatments have the capacity to increase basal plasma corticosterone levels in rats (Kitay, 1963). In addition, elevated plasma E_2 levels prolongs activation of the HPA axis with female rats having a delayed recovery of ACTH and corticosteroid secretion in response to stress compared to males (Burgess and Handa, 1992). Handa *et al.* (1994) suggests that elevated plasma ACTH and corticosterone levels may be secondary to a reduced female sensitivity to GCs at the pituitary level (via fewer GRs) which helps to minimize the damaging effects of prolonged GC exposure. Other E_2-sensitive areas, such as the hippocampus, have been implicated as influential regions affecting HPA activity in the rat (Turner, 1990).

As mentioned previously, the level of sexual maturity in teleosts moderates the activation of the HPI axis, not only in response to stress, but by regulating basal ACTH and cortisol secretion. This effect was clearly demonstrated by Pottinger *et al.* (1996) whereby the administration of E_2 elevated baseline levels of plasma ACTH and cortisol in immature rainbow trout (unstressed and acutely stressed) and brown trout (stressed), but had no effect on baseline levels in mature female fish under similar conditions.

Throughout their lives, rainbow trout and other commercially viable salmonid fishes are often presented with a number of environmental conditions, such as overcrowded aquaculture facilities, pollutants, and suboptimal water quality that serve as a source of stress to the fish. The

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impact of these conditions may play a significant role in the development of young fish since activation of the HPI axis in rainbow trout occurs very early in larval development. While Barry *et al.* (1995) found no evidence for such activity in response to a stress-induced rise in cortisol prior to and up to 2 weeks after hatching, interrenal tissue demonstrated an *in vitro* response to ACTH administration at hatching with a subsequent rise in cortisol levels after 3 to 4 weeks. These findings suggest that the final development of the stress response occurs at the level of the brain and requires a period of 3 to 4 weeks after hatching for the feedback loop of the HPI axis to reach maturation. This may be a period where the developing HPI axis of juvenile trout may be vulnerable to modification. The relationship between cortisol and E_2 secretion is obviously a very complex interaction. Whether the rise in plasma cortisol occurs during a stress response, or as a result of seasonal variation, it must be considered in light of the reproductive status, and also the gender of the fish. The fact that cortisol has the ability to the suppress plasma levels of both E_2 and its precursor, testosterone, makes it difficult to determine the extent to which stress impacts on the levels of E_2 in the CNS or how much E_2 ultimately reaches ERs within the HPG axis. The repercussions of chronically elevated plasma cortisol on ERs in the developing neurons of juvenile fish have yet to be investigated. To speculate, it seems apparent that the effects are to be far reaching, perhaps altering the fish's future reproductive capacity. Conversely, it seems that E_2 has the capacity to stimulate multiple levels of HPA axis activity and thereby enhance the endocrine response to stress by altering both GR-mediated and hormonal activity.

In summary, the findings presented in thesis independently support the existence of GRs and ERs at the level of the hypothalamus in the juvenile rainbow trout. Noteworthy is the fact that elements of both hypothalamic-pituitary axes are in place during the early development of these

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fish, thus the potential for one axis to impact on the other at such a time seems likely. While neither hypothalamic-pituitary axis can be fully understood as an independent system, the interplay between the HPI and the HPG axes in salmonid fishes is slowly being disclosed.

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

Kinetically derived equilibrium dissociation rate constant (K_d) was determined from association and dissociation experiments. $K_d = k_{-1}/k_{+1}$ was calculated from the association rate constant (k_{+1}) and dissociation rate constant (k_{-1}).

k_{-1} (min^{-1}) was estimated from the slope of the line obtained by plotting log B_{sp} versus time (hr) after the addition of a 5000 molar excess of radioinert competitor. B_{sp} is the specifically bound radioligand at time "t". The half-life ($t_{1/2}$) for the dissociation of specifically bound radioligand was calculated from $t_{1/2} = 0.693/k_{-1}$.

k_{+1} was estimated from $(k_{obs} - k_{-1})/[L]$ where [L] is the concentration of radioligand used. k_{obs} was estimated from the slope of the line obtained by plotting $\ln(B_{eq}/B_{eq} - B_{sp})$ versus time (hr) after the initiation of incubation. B_{sp} is the specifically bound radioligand at time "t". The slope of the line = k_{obs} (M^{-1} min^{-1}).

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Appendix B: Calculation of IC_{50}

Probability (P) of radioligand binding is based on the decimal fraction of maximum B_{sp} (P = 1.0) that corresponds to an IC_{50} value where P = 0.5. The linearization of logit-log transformed displacement data can be used to calculate the IC_{50} from the following equations:

$$
\begin{aligned}
y &= \ln (P/1.0-P) \\
&= \ln (0.5/1.0- 0.5) \\
&= \ln (1.0) \\
&= 0
\end{aligned}
$$

(Hulme and Birdsall, 1992)

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

where x corresponds to the log of the competitor concentration that results in 50% inhibition (IC_{50}) of radioligand that is maximally bound.

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Appendix C: Abbreviations

Word	Abbreviation
glucocorticoid	GC
glucocorticoid receptor	GR
hypothalamic-pituitary-interrenal	HPI
hypothalamic-pituitary-adrenal	HPA
hypothalamic-pituitary-gonadal	HPG
adrenocorticotropic hormone	ACTH
corticotropic releasing hormone	CRH
dexamethasone	DEX
17β-estradiol	E2
estrogen receptor	ER
gonadotropic hormone	GtH
gonadotropic releasing hormone	GnRH
central nervous system	CNS
γ-aminobutyric acid	GABA
serotonin	5HT
dopamine	DA
noradrenaline	NOR
adrenaline	ADR
B_O	Total binding
NSB	Nonspecific binding
B_{SP}	Specific binding
B_{eq}	Equilibrium binding
K_d	Dissociation constant/binding affinity
B_{MAX}	Maximum level of receptors
IC_{50}	Inhibitory concentration to obtain 50% B_{SP}
ligand	L
luteinizing hormone	LH
tricaine methanesulfonate	MS222
dextran-coated charcoal	DCC
11-deoxycortisol	11-DOC
triamcinolone	TA
standard error of the mean	SEM

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Appendix D: Structures of compounds used in the competitive displacement analysis.

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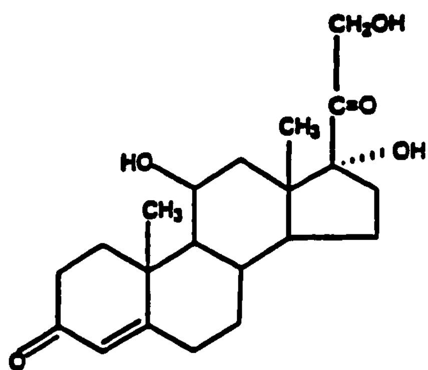
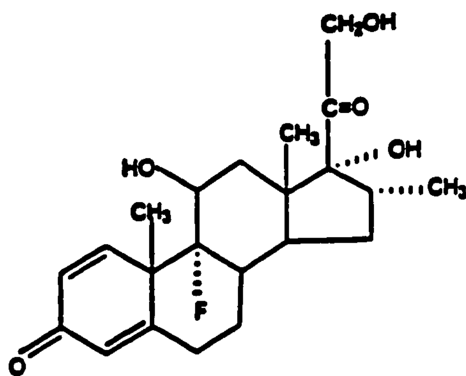
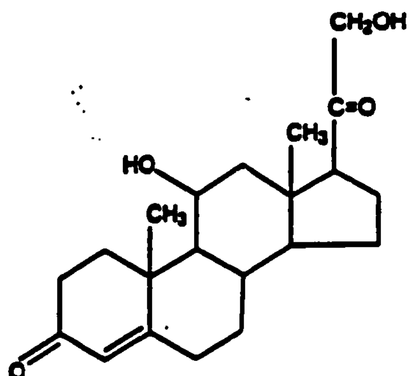


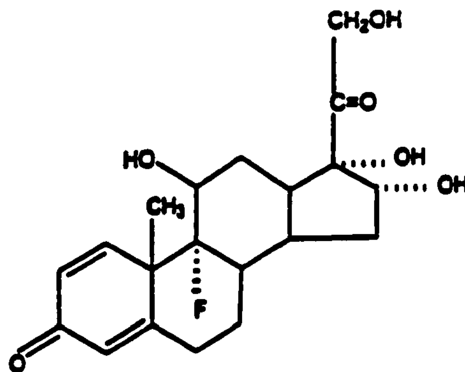
Cortisol



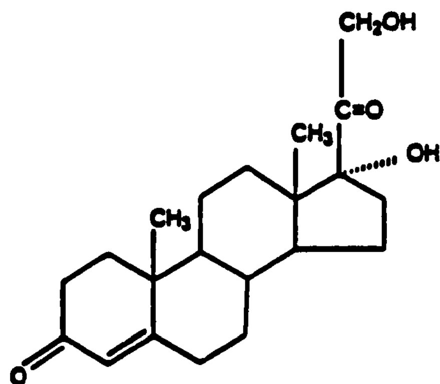
Dexamethasone



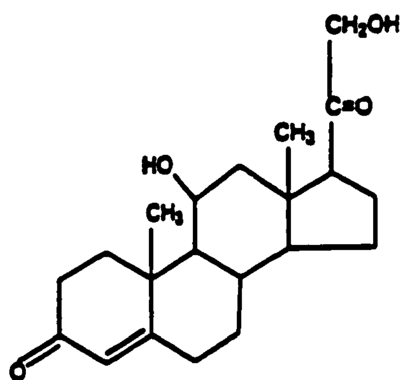
Corticosterone



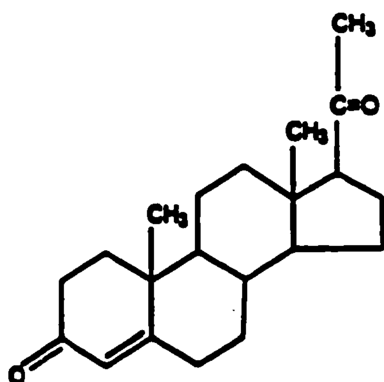
Triamcinolone



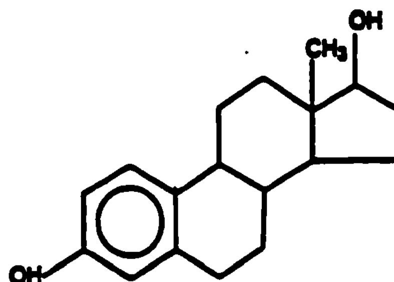
11-Deoxycortisol



Aldosterone



Progesterone



17β-Estradiol

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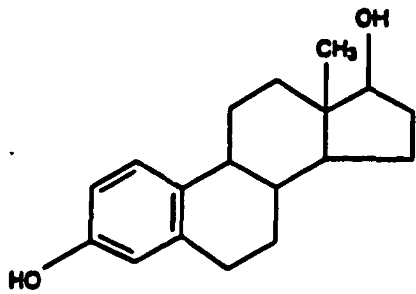
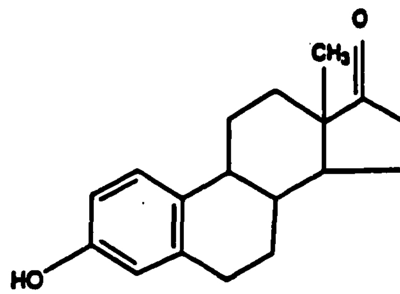
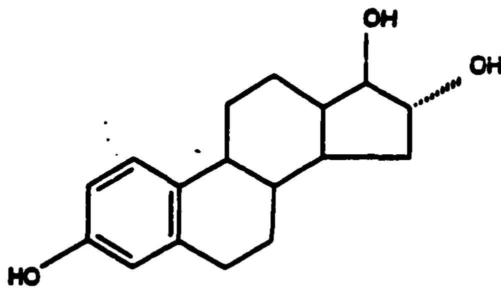


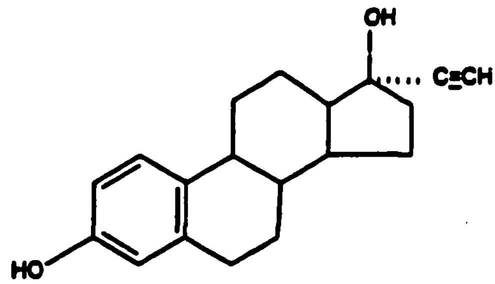
17β-Estradiol



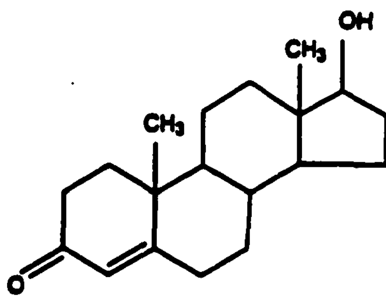
Estrone



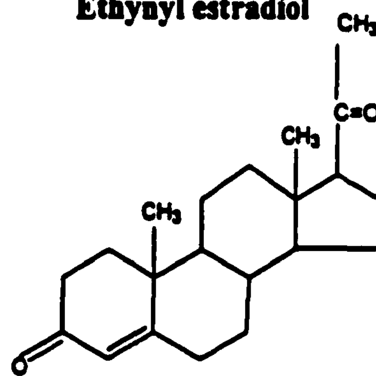
Estriol



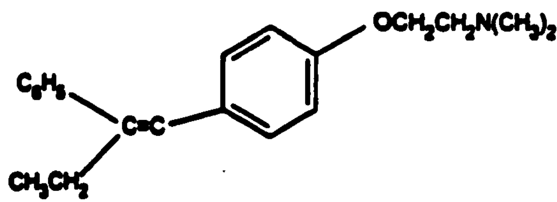
Ethynyl estradiol



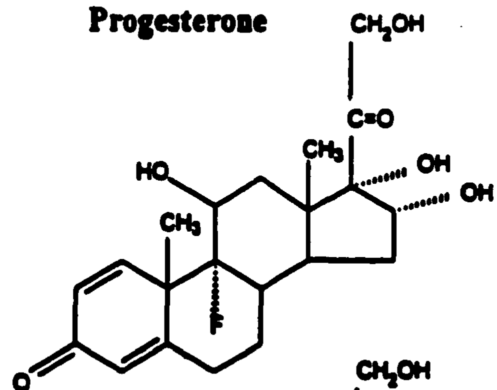
Testosterone



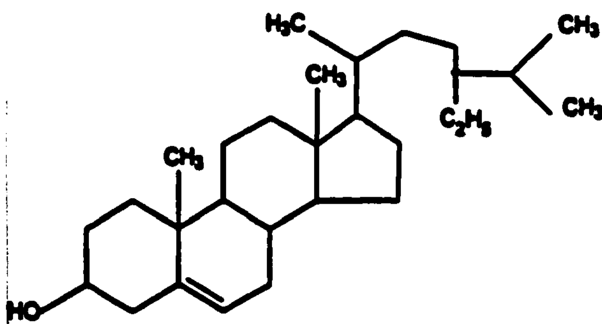
Progesterone

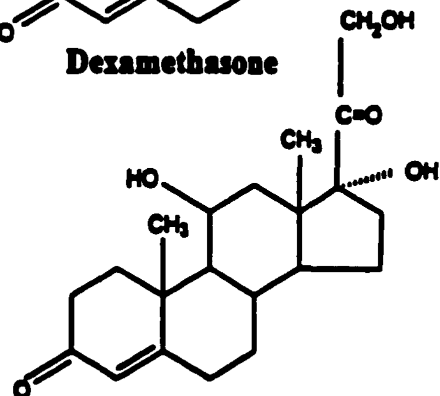


Tamoxifen



Dexamethasone





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