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Location and Characterization of Growth Hormone Binding Sites in the Central Nervous System of a Teleost Fish (*Oncorhynchus mykiss*)

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SUMMARY. The binding of ^{125}I -chinook salmon growth hormone (^{125}I -sGH) to rainbow trout brain membranes was studied. Specific binding was detected on telencephalon, thalamus-midbrain, cerebellum-medulla and hypothalamus. In all brain regions, specific binding was dependent on membrane protein concentration, being linear in the range of 250–2500 μg of membrane proteins (derived from 30–300 mg wet weight tissue). Scatchard analysis evidenced a single class of high affinity (8.2 ± 0.3 – $10 \pm 0.5 \times 10^9 \text{ M}^{-1}$) and low capacity (25.8 ± 6.1 – $62 \pm 4.5 \text{ fmol/g-tissue}$) GH-binding sites. Specific binding was competitively displaced by recombinant trout GH (rtGH) in a dose dependent-manner. Bovine GH appeared 40–50 fold less potent than cold rtGH for displacing ^{125}I -sGH. Chinook PRL, chinook GtH and bovine FSH did not compete for GH-binding sites in all brain regions examined. Binding studies performed in starved fish indicate that starving conditions decreases the binding of radiolabelled sGH to brain membrane preparations. Our results demonstrate the presence of specific and saturable GH-binding sites on the hypothalamic and suprahypothalamic areas of central nervous system. This finding supports the view that GH plays a direct role on the development and/or function of brain tissue in vertebrate species.

KEY WORDS: trout, brain, growth hormone, receptors, specificity, affinity physiological regulation.

INTRODUCTION

In teleosts, as in other vertebrate species, growth hormone has a pleiotropic action. It acts on growth¹ sea water adaptability,^{2–6} thyroid⁷ and corticotrop activity,⁸ and probably reproductive process.^{9,10} How GH modulates its own secretion remains to be elucidated, but it is likely that GH autoregulates its secretion through a short loop feedback mechanism, where GH mediates hypothalamic activity; or through a long loop feedback mechanism, where secretions from target tissues regulate GH release directly; and/or via hypothalamic system in a similar manner, as it has been demonstrated in higher vertebrates.^{11–13}

An important step in elucidating the mechanisms of GH action would be to locate GH receptors in target organs. In mammals and birds, binding sites for GH have been detected in several peripheral tissues, such as adipose tissue,¹⁴ liver,^{15,16} muscle,¹⁷ skin,¹⁸ cartilage¹⁹ and gonads.²⁰ The binding of GH

to central nervous system (CNS) has been examined without success by several authors.^{21,22} However high-affinity, low capacity binding sites have recently been evidenced by radioreceptor assay in chicken hypothalamus²³ and rabbit whole brain membrane preparations.²⁴ Furthermore, it has been observed that mRNAs extracted from hypothalamic and suprahypothalamic tissues of rabbit and chicken brains hybridize with a cDNA probe for GH receptors of rabbit liver,²⁴ remaining to establish a more suitable location and physiology of these receptors.

In fish, several tissues have been examined as a potential target tissue for GH binding, though saturable binding and specificity has only been demonstrated for hepatic binding sites. Purified teleost GHs have been employed to characterize hepatic binding sites in tilapia (*Sarotherodon mossambicus*),²⁵ coho salmon (*Oncorhynchus kisutch*)²⁶ Japanese eel (*Anguilla japonica*)²⁷ and rainbow trout (*Oncorhynchus mykiss*).²⁸ Recently, the authors have demonstrated the presence of saturable and specific binding sites for GH in trout testis.²⁹ Preliminary data also indicate the presence of GH binding in fish brain homogenates.^{26,28} Here, enriched-membrane preparations have been used to detect GH-binding sites in several

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regions of trout brain (telencephalon, thalamus-midbrain cerebellum-medulla, hypothalamus). The study demonstrates the presence of saturable and specific binding sites in all brain regions and also shows a diminished binding in starved fish.

MATERIALS AND METHODS

Experimental animals

(Characterization of GH receptors). Heads (900–1000) from freshly killed 2-year-old-rainbow trout were obtained from a local fish farm (Moulin de la Manche, Bretagne, France). The cranial cavity was opened dorsally and the whole brain was removed cutting the spinal cord and cranial nerves with fine scissors. The pituitary gland was left embedded in the sphenoid bone, while the brain section was placed in a petri dish, and cut into portions designated as telencephalon, thalamus-midbrain, cerebellum-medulla and hypothalamus (Fig. 1). Liver tissue was taken for comparative purposes.

Membrane preparations

Following dissection, each brain portion was placed in chilled assay buffer (20 mM Tris-HCl, 5 mM Mg₂Cl, 0.1% NaN₃, pH=7.5) containing PMSF 1 mM (5 g-tissue per 50 ml). The tissue was homogen-

ized using a Polytron homogenizer. The homogenate was passed through a cheesecloth and was further homogenized with a glass Teflon homogenizer. Aliquots (equivalent to 3 g wet weight of tissue) were placed into a discontinuous gradient of sucrose (25 ml) ranging between 0.3 and 1.2 M. Plasma membranes were collected at the 0.8/1 M sucrose interface after a high speed centrifugation (50 000 g for 90 min). Plasma liver membranes were homogenized in a similar way, but the final homogenate (suspended in sucrose 0.3 M) was centrifuged at 500 g for 10 min and the supernatant then centrifuged at 90 000 g for 90 min. The 90 000 pellet was kept to test its GH-binding. The protein content of brain and liver membrane preparations was determined by the method of BCA (Pierce), using bovine albumin as standard.

Hormones

Chinook salmon (*Oncorhynchus tshawytscha*) GH_{II} (sGH), recently purified by Le Bail et al.,³⁰ was used as a tracer. Chinook prolactin (sPRL) and chinook gonadotropin (sGtH) were purified as previously described Prunet and Houdebine³¹ and Breton et al.,³² respectively. Recombinant trout GH_{II} (rtGH) was supplied by Eurogentec SA (Liege, Belgium). Bovine GH (bGH) and bovine FSH (bFSH) were generously purchased from the National Institute of Health (NIH), Bethesda, Maryland.

Iodination

sGH was iodinated by the chloramine T method³³ with the Martal's³⁴ modification. Separation of ¹²⁵I-sGH from free ¹²⁵I was carried out by chromatography on a PD10 column (Pharmacia). Specific activity (SA=radioactivity content/protein content) was 55–65 µC/µg.

Binding assay procedure

A radioreceptor assay based on a previous hepatic GH-binding assay²⁸ was used. Briefly, plasma membranes (250–2500 µg of proteins derived from 30–300 mg wet weight tissue) and ¹²⁵I-sGH in assay buffer (20 mM Tris-HCl, 0.5% BSA, 0.1% NaN₃, with 0.25 mg/ml trypsin inhibitor and 0.5 mM acid ascorbic, pH=7.5) were incubated in triplicate for 20 h at 12°C in the absence (total binding) or presence (non-specific binding) of 2 µg rtGH in a final volume of 300 µl. Assay was terminated by adding 3 ml ice-cold assay buffer. Bound and free radioactivity was separated by centrifugation (3000 g for 15 min) and the pellets were counted in a Packard gamma counter (75% efficiency).

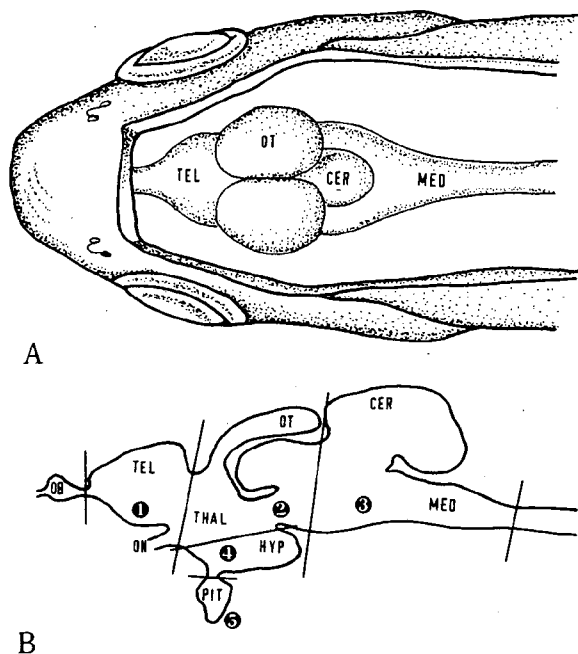


Fig. 1—(A) Diagrammatic section of rainbow trout skull showing a dorsal view of brain. (B) Planes of cuts used to dissect the brain into different regions. Designations of the regions are: 1, telencephalon; 2, thalamus-midbrain; 3, cerebellum-medulla; 4, hypothalamus. Abbreviations: OB, olfactory bulb; TEL, telencephalon; ON, optic nerve; OT, optic tectum; THAL, thalamus; HYP, hypothalamus; CER, cerebellum; MED, medulla; PIT, pituitary.

Physiological regulation of GH-binding sites

In order to investigate the effect of starvation on brain GH receptors, 100 1-year-old-trout (40–60 g weight) were acclimated to a recycling water unit for more than 1 month. The fish were distributed at random in two experimental tanks. One group was fed 'ad libitum', while the other group remained unfed throughout the experimental period (4 weeks). At the end of this period, brain tissue was removed from fish to test GH-binding. Plasma GH levels were determined by homologous salmon radioimmunoassay.³⁵

Statistics

Significant differences were estimated by parametric (Student t-test) and non-parametric (Kolmogorov-Smirnov) tests.

RESULTS

Before characterizing brain receptors, we considered two membrane preparation procedures: a) A serial centrifugation in sucrose 0.3 M where the 90 000 pellet was kept to test GH binding and b) a density gradient procedure where enriched-membrane fraction was collected at 0.8/1 M sucrose interface (see materials and methods). Figure 2 shows that non-specific binding remains unchanged (8–13% of total added counts, depending on the amount of tissue), but a significant loss of specific binding was observed in the serial

centrifugation. Taking into account this finding further membrane preparations were performed in the sucrose gradient way, where the non-specific binding accounts for >40% of total counts bound.

Figure 3 shows the binding of ¹²⁵I-sGH to increasing amounts of telencephalon, thalamus-midbrain, cerebellum-medulla and hypothalamus, expressed as a percentage of total counts added to each tube. Specific binding was dependent on membrane concentration, being linear in the range of 250–2500 µg of membrane proteins (derived from 30–300 mg of wet tissue). At the highest concentration, specific binding was 14.71 ± 0.18 for telencephalon, 11.55 ± 0.24 for cerebellum-medulla, 10.69 ± 0.10 for thalamus-midbrain and 9.04 ± 0.39 for hypothalamus. Under the same assay conditions the specific binding of ¹²⁵I-sGH to hepatic membranes was essentially linear up to 200 mg of tissue processed, then it reached a plateau which demonstrates that up to 50–60% of radiolabelled sGH may be bound to the receptors.

In all brain regions, specific binding was saturable by increasing doses of ¹²⁵I-sGH (Fig. 4). Scatchard³⁶ transformation was always linear, evidencing a single GH receptor population with a high affinity (K_a) and low capacity (B_{max}). Table 1 summarizes the binding characteristics of brain and liver membrane preparations. The binding affinities were of the same order of magnitude, ranging between $8.2 \pm 0.3 \times 10^9 \text{ M}^{-1}$ for cerebellum-medulla and $10.6 \pm 1.9 \times 10^9 \text{ M}^{-1}$ for liver. The abundance of the central GH-binding sites (suprahypothalamic-hypothalamic areas) was

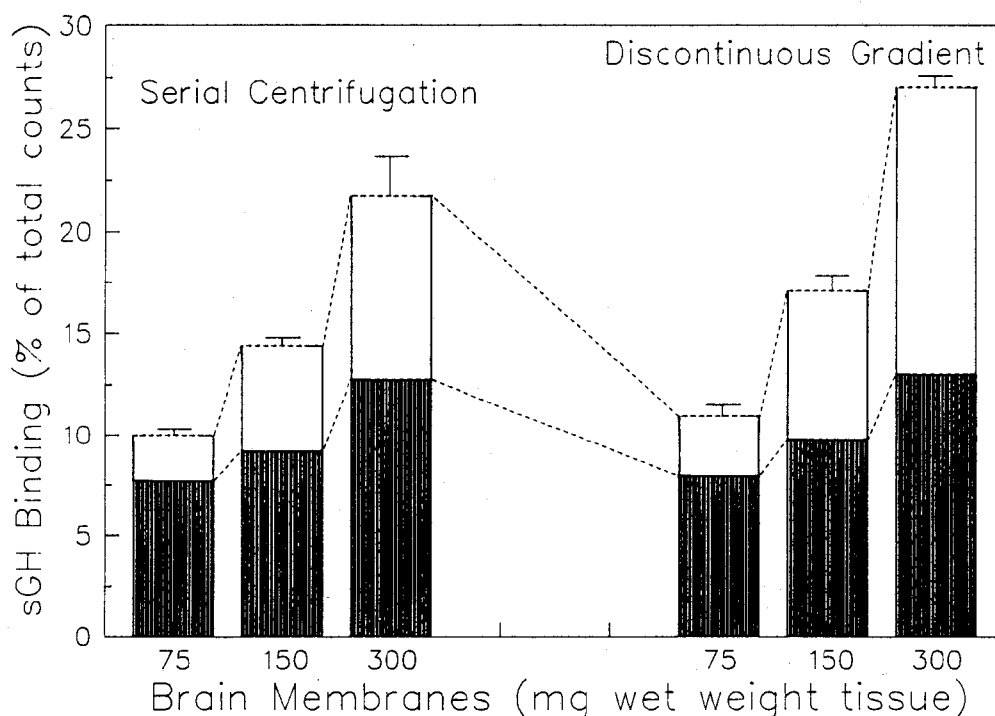


Fig. 2—Specific (□) and non-specific binding (■) of ¹²⁵I-sGH (10 000 cpm) to whole brain membranes obtained by serial centrifugation or discontinuous gradient of sucrose. Each histogram represents the mean \pm SEM of three determinations.

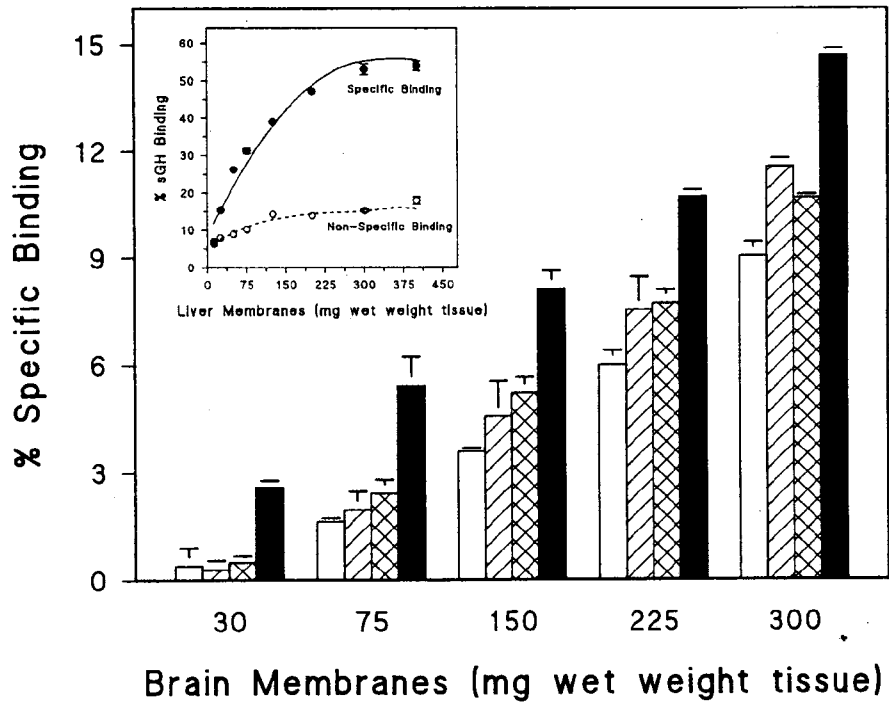


Fig. 3—Effect of the amount of hypothalamus (□), cerebellum-medulla (▨), thalamus-midbrain (▩) and telencephalon (■) on the specific binding of ¹²⁵I-sGH (20 000 cpm). The insert shows the binding of labelled sGH to hepatic membranes. Each value represents the mean ± SEM of three determinations.

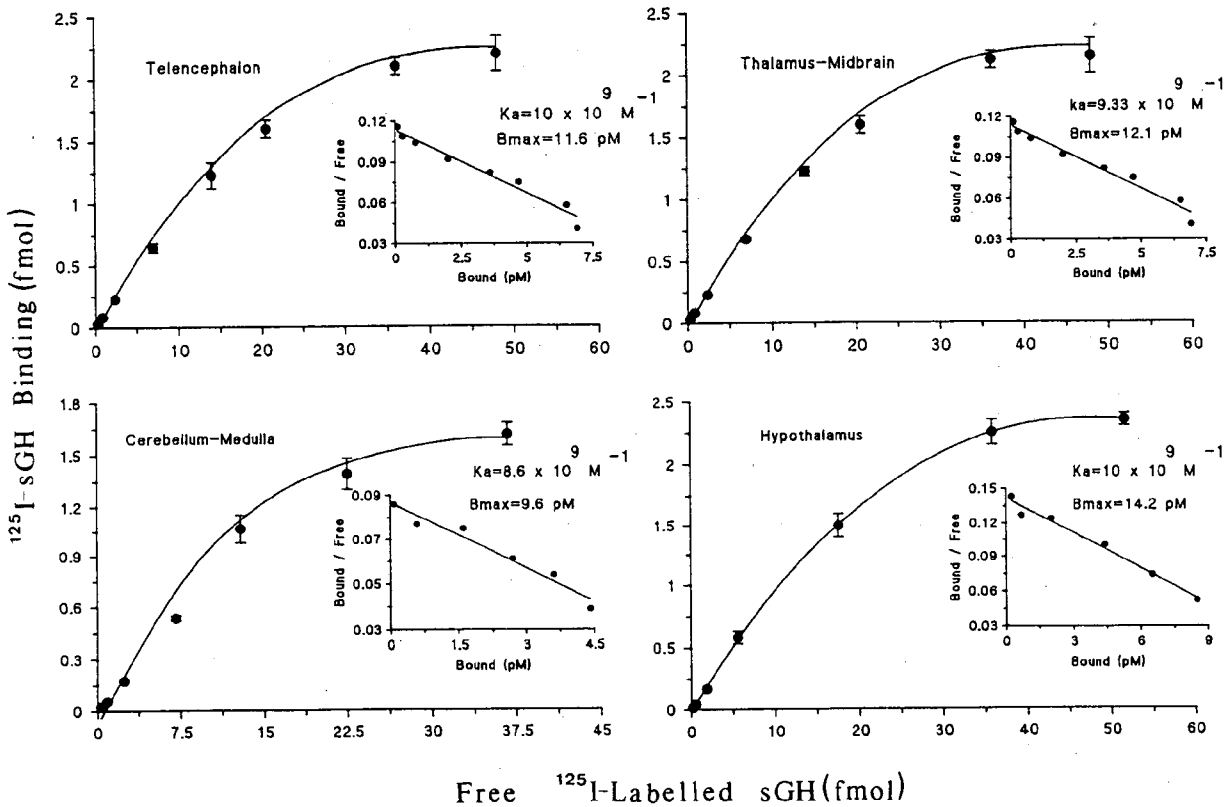


Fig. 4—Saturable specific binding of ¹²⁵I-sGH to brain membranes derived from 60 mg telencephalon, 100 mg thalamus-midbrain, 60 mg cerebellum-medulla and 130 mg hypothalamus. Each point represents the mean ± SEM of three-four replicates. The insert represents the derived Scatchard plot.

15–30 fold lower than that observed in hepatic membrane preparations (895.5 ± 150.2 fmol/g-tissue).

Figure 5 shows the results of competitive studies using a constant amount of ^{125}I -sGH and increasing doses of rtGH, bGH, sPRL, sGtH and bFSH. In all assays, specific binding was inhibited by rtGH in a dose dependent-manner. The relative potency of bGH was estimated in 40–50 fold lower than that of rtGH. The highest concentration of sPRL, sGtH and bFSH (125 ng per tube) did not inhibit the binding of ^{125}I -sGH to brain membrane preparations.

Figure 6 shows the effect of starving conditions on sGH binding. The non-specific binding appeared unaltered, but the specific binding (expressed as a percentage of the total radioactivity bound to 1 mg of membrane proteins in the treated pellets) of sGH to hypothalamic and suprahypothalamic areas of starved fish was lower ($p < 0.05$) than that observed in fed fish. This fact was linked to a significant decrease of body weight (15%), as well as to a significant increase ($p < 0.01$) in plasma GH levels from 4.08 ± 0.75 ($n = 41$) ng/ml in fed fish to 101.74 ± 13.64 ($n = 40$) ng/ml in starved fish.

DISCUSSION

In the present study, we used ^{125}I -labelled chinook GH as a tracer to demonstrate the presence of GH-

binding sites in the CNS of rainbow trout. Species differences in the affinity of GH preparations for GH-binding sites are well established.²⁵ Nevertheless, taking into account that trout and chinook GH sequences differ from each other by a single amino acid,^{37,38} we consider that our study was done in a homologous system.

Previous studies in chicken²³ and rabbit²⁴ species have demonstrated the presence of GH-binding sites in the CNS. In these earlier investigations, the authors employed a serial centrifugation in sucrose 0.3 M to obtain an enriched-membrane preparation, while we used a more adequate discontinuous gradient procedure (Fig. 2). In the present study, the maximum specific binding achieved a value of about 9–14% of total added counts (Fig. 3) which is higher than the 3 and 6% found for an equivalent amount of chicken hypothalamus and rabbit brain extract, respectively. Our increase in the measurable specific binding can be due to a greater abundance in GH-binding sites, though probably it also reflects a more suitable membrane preparation.

Scatchard analysis clearly demonstrate the presence of a single class of binding sites in each of the four brain regions examined (telencephalon, thalamus-midbrain, cerebellum-medulla and hypothalamus) (Fig. 4). The binding affinity is comparable to that previously observed in chicken hypothalamus²³ and rabbit brain.²⁴ It is also equivalent to that observed

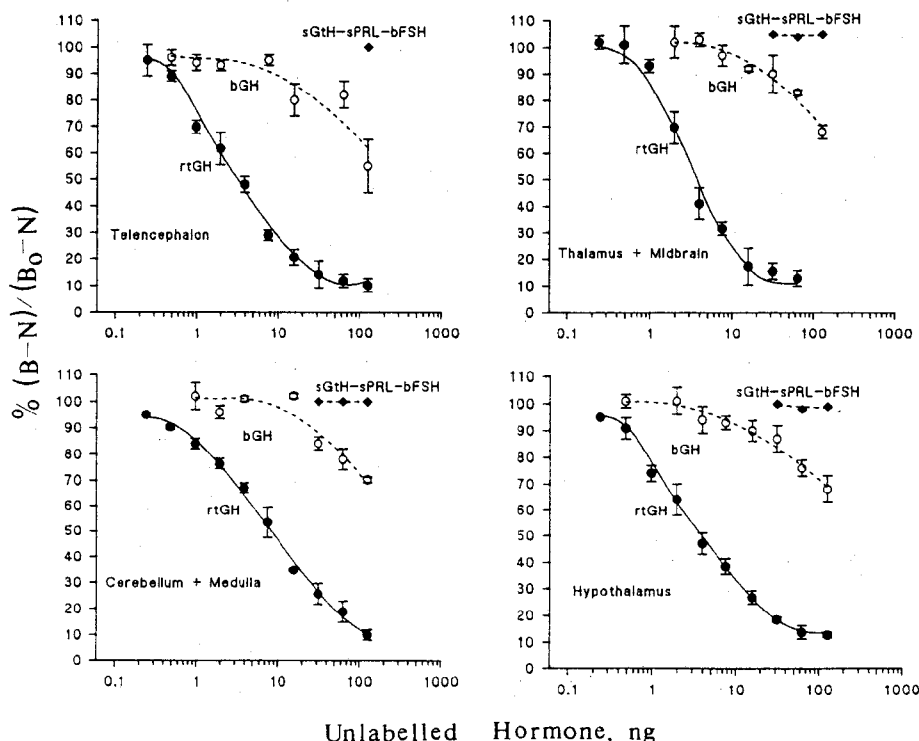


Fig. 5—Competitive displacement of ^{125}I -sGH (35 000 cpm) from brain membranes derived from 80 mg telencephalon, 110 mg thalamus-midbrain, 130 mg cerebellum-medulla and 130 mg hypothalamus. B_0 represents the labelled sGH bound in the absence of unlabelled hormone (rtGH, bGH, sPRL, sGtH and bFSH), B represents the labelled sGH bound in the presence of a given amount of the unlabelled hormone and N represents the labelled sGH bound in presence of 2 μg of rtGH. Each value represents the mean \pm SEM of four replicates.

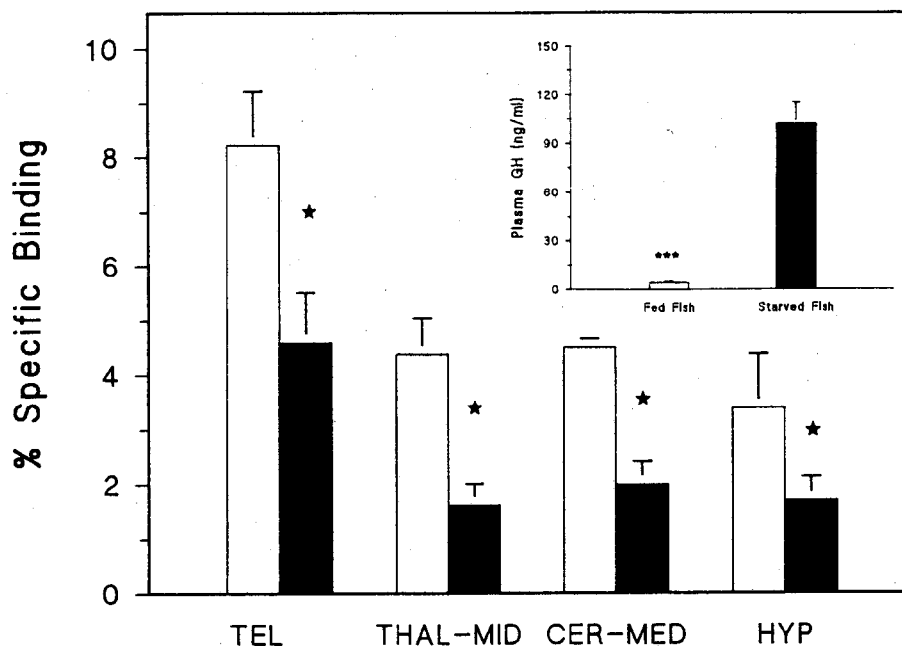


Fig. 6—Specific binding of ^{125}I -sGH to hypothalamic and suprahypothalamic membranes (1 mg membrane protein per tube) of starved (■) and fed fish (□). Abbreviations: Tel, telencephalon; THAL-MID, thalamus-midbrain; CER-MED, cerebellum-medullar; HYP, hypothalamus. Each histogram represents the mean \pm SEM of five determinations (*, $p < 0.05$, Kolmogorov-Smirnov). The insert shows plasma GH levels. Each value is the mean \pm SEM of 40–50 determinations (***, $p < 0.001$, Student t-test).

in mammalian,^{15,16} avian³⁹ and fish hepatic membranes, as this (Table 1) and previous studies^{25,28} indicate. As in chicken and rabbit species, the number of central binding sites is far lower than that found in liver tissue, considered as the most important target for the direct action of GH.⁴⁰ However, the potential binding of GH to brain membranes could be ignored, since it is quite possible that this low abundance in GH-binding sites reflects an uneven distribution throughout the CNS. Interestingly, we saw a relative concentration of GH-binding sites on the telencephalon, which in lower vertebrates integrates taste, olfactory, visual, auditory and somatosensory and visceral information.⁴¹ At least in fish species, this concentration in central GH-binding sites appears to be corroborated by a recent observation of Gray et al.²⁶ These authors showed a poor but significant GH binding in the anterior region of coho salmon brain (including telencephalon), but did not detect GH binding in middle and posterior brain regions.

The results of the specificity studies showed the limited potency of bGH in displacing ^{125}I -sGH from

trout brain (hypothalamic and suprahypothalamic tissues) (Fig. 5). Similarly, previous investigations have demonstrated the poor affinity of mammalian GHs for the hepatic GH-binding sites,^{25,26,28} which suggests important differences between mammalian and fish GH-binding sites. Some affinity of sPRL for sGH-binding sites might be expected since GH and PRL have evolved from a common ancestral molecule,⁴² but the cross-reactivity of sPRL was negligible. This, together with the observation that more dissimilar polypeptide hormones (sGtH and bFSH) did not compete for brain GH-sites, demonstrates a high hormonal specificity.

The binding properties (finite number of binding sites, high affinity, hormone specificity) of sGH to trout brain provide conclusive evidence of the presence of hypothalamic and suprahypothalamic GH receptors in the CNS of fish species, as has been previously suggested for birds and mammals. The question is how pituitary GH reaches these binding sites. The synthesis of GH outside the pituitary has not been demonstrated in fish and the passage of GH across the blood-brain barrier appears unlikely.⁴³ However, there is anatomical basis to consider a retrograde flow from the pituitary to hypothalamus,^{44,45} whereby pituitary hormones may reach hypothalamic and extrahypothalamic sites within the brain. This vascular system shows phylogenetic constancy, as it has been found in mammals, birds, reptiles, amphibians and elasmobranch fish. In teleosts, it appears less developed, probably due to the fact that secretory cells of pituitary are directly (or almost directly) innervated by hypothalamic nerve

Table 1. Binding affinities (K_a) and binding capacities (B_{max}) of CNS and liver tissue. All values are mean \pm SEM of () separate determinations.

	K_a (M^{-1})	B_{max} (fmol/g-tissue)
Telencephalon	$10 \pm 0.5 \times 10^9$ (2)	62.3 ± 4.5 (2)
Thalamus-Midbrain	$8.2 \pm 1.1 \times 10^9$ (3)	53.2 ± 3.7 (3)
Cerebellum-medulla	$8.2 \pm 0.3 \times 10^9$ (3)	42.1 ± 1.1 (3)
Hypothalamus	$9.8 \pm 0.2 \times 10^9$ (2)	25.8 ± 6.1 (2)
Liver	$10.6 \pm 1.9 \times 10^9$ (5)	895.5 ± 150.2 (5)

fibers.⁴⁶ Nevertheless, it could be also effective to establish an useful retrograde flow.

From a functional point of view, it is interesting to underline that a significant amount of plasma GH levels was observed in starved trout, as it has been previously reported by Barret and Mckeown⁴⁷ and Sumpter et al.⁴⁸ This increase was linked to a decrease of sGH binding to hypothalamic and suprahypothalamic membranes (Fig. 6). Conversely, in chicken and rabbit brain, the age-related decrease in plasma GH levels appears linked to a greater expression of GH-receptor mRNA.²⁴ Endogenous saturation and/or down-regulation of GH receptors by high GH levels may explain the low sGH binding in starved fish,⁴⁹ though we cannot ignore that in rats bearing GH-secreting tumors or receiving chronic GH infusions, GH receptors are up-regulated.^{50,51} Alternatively, it is likely that this decreased binding reflects a diminished short loop feedback activity, since the central inhibitory effect of GH on its own secretion may occur by binding sites within the brain. The fact that GH enhances the release of somatostatin from rat hypothalamus,^{52,53} as well as the inhibition of GH release following intracerebroventricular administration of GH in rat⁵⁴ and chicken,⁵⁵ supports this view. It is also possible that the decrease in sGH binding occurs as a metabolic adaptation to the decline in nutrient availability. This hypothesis is consistent with the observation that GH has central effects on brain size and on its DNA, protein, amine and ornithine decarboxylase content and even on behavior patterns (e.g. Zamenhof et al⁵⁶; Sara et al⁵⁷; Roger et al⁵⁸; Ster et al⁵⁹; Drucker-Collin⁶⁰).

In conclusion, this work confirms the presence of GH receptors on hypothalamic and suprahypothalamic areas of a vertebrate species. A decrease in central GH binding is observed in starved fish, but further research is needed to establish the location and contribution of central GH receptors on the physiological action of GH.

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