Growth Hormone Receptor Messenger Ribonucleic Acid Distribution in the Adult Male Rat Brain and its Colocalization in Hypothalamic Somatostatin Neurons*

KIMBERLY A. BURTON, EMILIA B. KABIGTING, DONALD K. CLIFTON, AND ROBERT A. STEINER


ABSTRACT

The activity of both somatostatin (SS) and GH-releasing hormone (GHRH) neurons within several hypothalamic nuclei is regulated, in part, by the feedback effects of GH. However, whether GH, or its intermediate, insulin-like growth factor I, acts on these neurons to alter the synthesis and release of SS and GHRH is unknown. We argued that if GH itself acts directly on the brain to govern its own secretion, then regions of the brain containing SS and GHRH neurons may express the GH receptor gene. We tested this hypothesis by performing in situ hybridization for GH receptor messenger RNA (mRNA) and mapping its distribution in the brain. We observed GH receptor mRNA-containing cells in various brain regions including the hypothalamus, septal region, hippocampus, dentate gyrus, amygdala, and hypothalamus. Next we sought evidence for expression of the GH receptor mRNA by SS neurons in the hypothalamus. We addressed this by performing a double-label in situ hybridization to identify neurons expressing both SS mRNA and GH receptor mRNA. We report that SS neurons in the periventricular nucleus and in the paraventricular nucleus coexpress the GH receptor gene, whereas few, if any, of the SS neurons in the cortex express detectable amounts of the GH receptor mRNA. These findings suggest that GH acts directly on the brain and participates in the regulation of its own secretion through a direct action on hypothalamic SS neurons. (Endocrinology 130: 958-963, 1992)

IN THE male rat, GH secretion is pulsatile with peaks in plasma GH levels occurring approximately every 3 h superimposed on a low, or undetectable, baseline (1). This pattern of GH secretion is generated by the reciprocal action of two hypothalamic peptides, somatostatin (SS) and GH-releasing hormone (GHRH). SS, secreted from neurons located in the periventricular nucleus (PeN) and paraventricular nucleus (PVN) (2, 3), inhibits GH release, whereas GHRH, secreted from neurons in the arcuate nucleus (4), stimulates GH release (5). SS and GHRH neurons are thought to be regulated, in turn, by the feedback effects of GH (6–13). Although the basic elements of this control system have been identified, we understand little about the molecular and cellular mechanisms by which GH exerts its effects on these neurosecretory cells in the brain.

There are at least two possible mechanisms by which GH could influence the activity of SS and GHRH neurons. GH could act indirectly through an intermediary, such as insulin-like growth factor-I (IGF-I), which, in turn, would transduce its effect on the brain through the IGF-I receptor. Several lines of evidence argue that this mechanism is plausible. First, the central administration of IGF-I suppresses the release of pituitary GH (14, 15), an effect thought to be generated, in part, by an increase in the release of SS from the hypothalamus (16). Second, IGF-I receptor messenger RNA (mRNA) is located in the brain (17), and IGF-I binding sites are found in the hypothalamus and median eminence, as well as other brain regions (18–20), suggesting that IGF-I participates in the central feedback effects of GH.

The evidence for IGF-I action on the brain does not preclude a direct action by GH itself on the brain. In fact, this has been suggested by the results of several studies. Centrally administered GH suppresses the endogenous release of GH, presumably by either stimulating SS or inhibiting GHRH release (21). In addition, GH receptor mRNA has been found in whole rat brain by Northern blot analysis (22), and immunoreactive GH receptor has been identified in various regions of the 10-day-old rat brain, including the hypothalamus (23). These studies provide evidence for a direct effect of GH on the brain but leave unresolved the identity and location of target cells mediating the action of GH.

We argued that if GH were to act directly on the central nervous system to regulate its secretion, neurons in regions of the brain known to govern GH secretion should express the GH receptor gene. We had two objectives in performing this study. The first was to map the neuroanatomical distribution of GH receptor mRNA in the adult male rat brain, and the second was to determine whether hypothalamic SS neurons coexpress the GH receptor gene.

Materials and Methods

Animals

Adult male Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA) and housed under constant light (14 h)-dark...
(10 h) conditions with lights on at 0600 h. The animals had free access to rat chow and tap water.

**Tissue preparation**

Animals were killed by asphyxiation with carbon dioxide and immediately decapitated. The brains were rapidly removed, frozen on dry ice, and stored at -80 °C. Coronal brain slices (20 μm) were cut with a cryostat, thaw-mounted onto poly-L-lysine (50 μg/ml)-coated slides, and stored at -80 °C in airtight boxes. Using the rat atlas of Paxinos and Watson (24) as an anatomical guide, we collected coronal slices beginning rostrally at the joining of the anterior commissure and continuing caudally through the dorsomedial nucleus.

**Probe preparation**

A complementary DNA (cDNA) clone, pG0.9, containing the 900-base pair (bp) BglII fragment of the rat GH receptor cDNA cloned into the BamHI site of the vector of the pGEM-3 plasmid vector, was kindly given to us by Dr. L. S. Mathews (presently at The Salk Institute, La Jolla, CA) and Professor G. Norstedt (The Huddinge University Hospital, Huddinge, Sweden). The extracellular domain of the GH receptor includes this 900 bp fragment. Therefore, this cDNA fragment should recognize both alternatively spliced products of the GH receptor gene, i.e., the membrane-spanning receptor and the circulating binding protein (22). Anti-sense cRNA probes were synthesized in vitro with T7 polymerase on plasmid DNA linearized with HindIII. The sense cRNA probes were made in vitro with T3 polymerase on plasmid DNA linearized with EcoR1. The cRNA probe was synthesized in vitro with [53S]cystidine triphosphate (UTP) (New England Nuclear, Boston, MA) at a concentration of 50 μM. Residual DNA was removed by digestion with DNase (Promega Biotec, Madison, WI), and the cRNA probe was separated from unincorporated nucleotides by gel electrophoresis. One base-pair fragment of transfer RNA. We determined in preliminary studies that a GH receptor cRNA probe concentration of 0.075 pg/kilobase (kb) ml was necessary to saturate the hybridization reaction in hypothalamic tissue and, therefore, a final concentration of 0.10 pg/kb.ml with a specific activity of approximately 2.5 x 10⁹ dpm/pg was used for the experimental tissue. Since the exact yield of the transcription reaction with digoxigenin-UTP for the SS cRNA probe cannot be derived, a test-label in situ hybridization to the tissue. These control experiments have been performed previously for the SS probe (10). First, a 3S-labeled sense probe was placed on tissue. Second, 100-fold greater concentration of unlabeled antisense probe in the presence of 3S-labeled antisense probe (at a concentration of 0.1 ng/ml.kb) was placed on the tissue. The tissue was processed for hybridization histochemistry as described above. Both experiments resulted in complete loss of photographic grain clusters over cells and suggested that the 3S-labeled probe was binding to a specific sequence in the GH receptor mRNA (data not shown).

**Control experiments**

Two control experiments were performed to assess the specific binding of the GH receptor riboprobe to the tissue. These control experiments have been performed previously for the SS probe (10). First, a 3S-labeled sense probe was placed on tissue. Second, 100-fold greater concentration of unlabeled antisense probe in the presence of 3S-labeled antisense probe (at a concentration of 0.1 ng/ml.kb) was placed on the tissue. The tissue was processed for hybridization histochemistry as described above. Both experiments resulted in complete loss of photographic grain clusters over cells and suggested that the 3S-labeled probe was binding to a specific sequence in the GH receptor mRNA (data not shown).

**Semi-quantitative analysis of cellular mRNA**

Tissue sections were viewed under a Zeiss Axioskop (Zeiss, New York, NY) equipped with a 40X epi-illumination darkfield objective. To estimate the percentage of SS cells coexpressing the GH receptor gene, a grain counting program was used to measure the number of silver grains overlying SS cells in the PeN, cortex, and the PVN. A purple-stained digoxigenin-labeled SS cell was first isolated under brightfield illumination. The silver grains overlying the cell were then visualized under darkfield epi-illumination. We determined the number of silver grains per cell using a grain counting program as previously described (29). This system consisted of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Macintosh IIIx computer (Apple Computer, Cupertino, CA). Video images were obtained by a Dage model 65 camera (Dage-MTI, Inc., Michigan City, IN) attached to the Zeiss Axioskop.

Twelve tissue sections per animal (n = 3) which were equally spaced
throughout the PeN were analyzed for the number of grains per cell overlying SS cells in the PeN. In these same tissue sections, 10 randomly chosen SS cells per tissue section were measured in the cortex for the number of silver grains overlying each cell. Likewise, grain counting was performed on 6-10 tissue sections per animal over SS cells located in the PVN.

How are these grain counts used to determine whether SS neurons coexpress the GH receptor mRNA? First, it should be noted that when grains are counted over a population of cells, a range of values is obtained. If the message being measured is expressed at low levels (as is the GH receptor message), the frequency distribution of grains per cell counted over specifically labeled cells overlaps the distribution of grain counts found over unlabeled cells. This overlap makes it difficult to estimate accurately the number of coexpressing cells. To address this problem, we sought to establish a threshold value that could be used to differentiate between cells that unequivocally contain the GH receptor message (as indicated by the presence of many grains over their soma) and cells with grain counts that are too low for us to be certain that they express the GH receptor gene. To derive the appropriate threshold value, we first determined the frequency distribution of grains over cells not thought to express the GH receptor gene. For this, we used the frequency distribution of grain counts over SS mRNA-containing cells in the cortex, since we had established with the single-label GH receptor mRNA experiments that the GH receptor gene is not expressed in detectable quantities in the cortex. We set a threshold that was equal to or greater than 95% of the grain counts over cortical SS cells; any cell with a grain count higher than this value was accepted as a GH receptor mRNA-positive cell.

### Results

**GH receptor mRNA distribution**

The GH receptor mRNA was located in distinct cell populations in the adult male rat brain, as listed in Table 1. The GH receptor mRNA was observed in the thalamus, septal region, hippocampal formation, amygdala, and hypothalamus. In the hypothalamus, GH receptor mRNA was localized in the PeN, the PVN, and the arcuate nucleus, as shown in Fig. 1, as well as in the dorsomedial nucleus and medial tuberal nucleus. The GH receptor mRNA was observed throughout the rostral-caudal extent of the PeN. In the PVN, the GH receptor mRNA was localized to the anterior and medial parvocellular part and the periventricular part (29).

**Colocalization of GH receptor mRNA and SS mRNA**

When the brain sections were processed through the double-label in situ hybridization protocol for SS mRNA and GH receptor mRNA, GH receptor mRNA was colocalized with SS mRNA in neurons in the PeN (as shown in Fig. 2A) and in the PVN. There was no evidence for the presence of GH receptor mRNA in cortical SS neurons (as shown in Fig. 2B).

Grain counting was performed on silver grains overlying purple-stained digoxigenin-labeled SS cells in the PeN and the PVN, and means of 25 ± 3.7 and 31 ± 3.5 grains per cell were observed in these nuclei, respectively. The frequency distributions of these grain counts are shown in Figs. 3 and 4. As shown in Fig. 4, the frequency distribution of grains per cell overlying cortical SS cells (mean of 2 ± 0.6) confirmed that either this population of SS neurons does not express the GH receptor gene or does so at a level below the detectability of the assay. The threshold for detecting GH receptor mRNA-containing cells was determined from this distribution (see Materials and Methods for details). Ninety-five percent of the cortical SS cells contained 16 grains per cell or fewer; therefore, we used 16 grains per cell as the threshold for positive identification of GH receptor mRNA-containing cells. Sixty-nine percent of the SS cells in the PeN and 72% in the PVN contained more than 16 grains per cell. This suggests that at least 69% of the SS cells in the PeN and 72% in the PVN coexpress the GH receptor gene.
Discussion

In this study, we have demonstrated that the GH receptor mRNA is expressed in distinct cell populations in the adult male rat brain. Furthermore, we have shown that the GH receptor mRNA is colocalized in a subset of SS mRNA-containing cells in the PeN and PVN of the hypothalamus. These findings suggest that GH acts directly on the central nervous system to regulate its own secretion by a direct negative feedback action on SS neurons in the hypothalamus.

Our data complement and extend earlier reports on the presence of GH receptor protein and mRNA in the rat brain. It has previously been shown that in the 10-day-old rat brain, immunoreactive GH receptor is localized in several areas including the thalamus, hippocampus, dentate gyrus, and hypothalamus (23), and in the 6-week-old male rat, GH receptor mRNA is found in the brain by Northern blot analysis (22). Our data support these findings and provide additional information on the cellular localization of the site of GH receptor synthesis in the adult rat brain. Similar to the distribution of the GH receptor protein, we observed that the GH receptor mRNA is found in the thalamus, hippocampus, dentate gyrus, and the hypothalamus. Furthermore, we observed the GH receptor mRNA in the amygdala, lateral septum, and the bed nucleus of the stria terminalis, areas which do not apparently contain the GH receptor protein. This discrepancy could be due to either developmental changes in the expression of the GH receptor gene (10 days vs. adult), or methodological differences between the two studies [in situ hybridization vs. immunocytochemistry (ICC)]. The receptor protein that is labeled by ICC may be located distal to a somatic site of protein synthesis and GH receptor message accumulation as identified by in situ hybridization. Also, in situ hybridization for GH receptor message may be more sensitive than the immunocytochemical procedure for GH receptor protein, permitting us to detect GH receptor-containing cells that remain unlabeled by ICC.

Although our results permit us to unequivocally affirm the existence of GH receptor mRNA in SS mRNA-containing cells, relatively low message levels and the lack of a clear bimodal distribution of grains counted over SS cells make it impossible to determine whether all SS neurons express the GH receptor gene. Based on the distribution of grain counts over SS neurons in the cortex (an area which apparently

Fig. 2. SS mRNA-containing cells and GH receptor mRNA-containing cells in the PeN (A), and in the cortex (B). GH receptor mRNA-containing cells are marked by silver grain clusters (clusters of white dots), and SS mRNA-containing cells appear as dark colored cell bodies. In A arrows indicate cells double-labeled for both SS mRNA and GH receptor mRNA. In B arrows indicate cells that appear to contain only SS mRNA. There appear to be no GH receptor mRNA-containing cells in the frontal cortex.
GH RECEPTOR mRNA IN RAT BRAIN

Our observations that GH receptor mRNA is colocalized in SS mRNA-containing cells in the PeN and PVN reinforce earlier physiological studies demonstrating that GH has direct effects on hypothalamic SS synthesis and release. GH stimulates the release of hypothalamic SS (9, 11, 12), and increases hypothalamic SS content in the hypothalamus (6, 7) and SS mRNA in the PeN (10). Given that SS neurons in both the PeN and PVN project to the median eminence (2, 3), our current finding that the GH receptor mRNA is present in SS neurons in these nuclei suggests that GH participates in a short-loop feedback regulation of its own secretion. This direct feedback action by GH is further suggested by our preliminary findings that the GH receptor mRNA is also expressed in some GHRH neurons in the arcuate nucleus (30). This short-loop feedback action by a GH receptor-dependent mechanism on hypothalamic SS and GHRH neurons may be an important step in the regulation of GH secretion.

We observed that the GH receptor gene was expressed in cells which do not contain SS mRNA. These unidentified cells expressing the GH receptor gene were observed outside the hypothalamus, such as in the hippocampal formation, as well as within the hypothalamus. The role of the GH receptor in these unidentified cells remains to be elucidated. However, within the hypothalamus, the close proximity of GH receptor mRNA-containing cells to SS and GHRH neurons and other important neuroendocrine cells suggests that the unidentified GH receptor-containing cells may also play a role in transducing the effects of GH on those cells which govern the secretion of the pituitary.

The presence of the GH receptor mRNA and protein in the brain suggests that GH, derived from either the pituitary or possibly the brain itself (31, 32), binds to these receptors. The receptors in SS neurons may be located on nerve terminals (i.e. median eminence) outside of the blood-brain barrier, in which case, GH of pituitary origin could have ready access to SS target cells. It's also possible that the receptors are located on cell bodies within the privileged blood-brain barrier. How could GH of pituitary origin gain access to GH receptors on SS neuronal cell bodies? It may be possible that GH enters the brain by crossing the blood-brain barrier. Although the ability of GH to pass through the blood-brain barrier has not been demonstrated, other hormones, including insulin, have been shown to be transported across the blood-brain barrier (33). Moreover, the presence of receptors for several blood-borne proteins including IGF-I and II have been localized in the capillaries of the brain (33). These receptors presumably act as either transporters or as classical mediators of hormone action in brain capillaries. It remains to be determined whether such a transporter exists for GH in the blood-brain barrier.

In summary, our results demonstrate that the GH receptor mRNA is expressed in discrete cell populations in the adult male rat brain and that it is colocalized in SS mRNA-containing neurons in the PeN and PVN. These observations...
suggested that GH acts directly on the brain and that the negative feedback action of GH occurs through its own receptor expressed in SS neurons in the hypothalamus.

Acknowledgments

The authors are grateful to Lawrence Mathews and Gunnar Norstedt for the GH receptor cDNA and Richard Palmer for the SS cDNA. We are also indebted to Daniel Marks for his help in establishing the double-label in situ hybridization protocol.

References

30. Burton KA, Steiner RA, Clifton DK, Double-label in situ hybridization confirms that the GRF gene and the GH receptor gene are coexpressed in a subset of neurons in the arcuate nucleus. Program of the 74th Annual Meeting of The Endocrine Society, San Antonio, 1992 (Abstract)
32. Gossard F, Dihl F, Pelletier G, Dubois PM, Morel G 1987 In situ hybridization to rat brain and pituitary gland of growth hormone (DNA, Neuropeptide Lett 79:251-257