Endocrine, *vol. 14, no. 1, 15–19, February 2001* 0969–711X/01/14:15–19/$11.25

Central Actions of the Nonpeptide Growth Hormone Secretagogue GHS-25

Alex R. T. Bailey,[1] Louise Gilliver,[2] Gareth Leng,[2] and Roy G. Smith[1]

[1]Huffington Center on Aging, Baylor College of Medicine, Houston, TX; [2]Department of Biomedical Sciences, University Medical School, Edinburgh, EH8 9AG, UK.

Growth hormone secretagogues (GHSs) increase the activity of hypothalamic arcuate nucleus neurons thought to be involved in controlling the release of growth hormone (GH). The GHS receptor is also found in hypothalamic regions not associated with the release of GH, suggesting that GHSs may influence other hypothalamic systems. This study utilized double-labeling immunocytochemical techniques to examine the hypothalamic actions of a novel nonpeptide GHS, GHS-25. In common with other GHSs, GHS-25 induced significant amounts of Fos immunoreactivity in the arcuate nucleus of conscious male rats. However, unlike other GHSs, GHS-25 also induced Fos immunoreactivity in the supraoptic nucleus. Double labeling revealed that approx 66% of supraoptic nucleus cells that were Fos positive after the administration of GHS-25 were also immunoreactive for oxytocin. Thus, in addition to its actions on the GH axis, GHS-25 may influence the release of neurohypophyseal hormone.

Key Words: Hypothalamus; arcuate nucleus; supraoptic nucleus; oxytocin; vasopressin.

Introduction

Growth hormone (GH) release can be elicited directly from the anterior pituitary gland by growth hormone secretagogues (GHSs), such as GHRP-6 and MK-0677 *(1,2)*. In addition to inducing GH release directly from the pituitary, GHSs act centrally to increase Fos protein expression in the hypothalamic arcuate nucleus and induce a prolonged increase in the electrical activity of neurosecretory arcuate nucleus neurons *(3,4)*. Within the arcuate nucleus, the main populations of GHS-responsive cells are thought to include both GH-releasing hormone (GHRH) and neuropeptide Y (NPY) cells *(5)*. Indeed, GHS mRNA has recently been localized to GHRH and NPY arcuate nucleus neurons *(6,7)*. However, GHS receptor mRNA is also found in several other hypothalamic nuclei (e.g., the supraoptic nucleus [SON] and suprachiasmatic nucleus [SCN]), and in other discrete regions of the brain, including the CA2 and CA3 regions of the hippocampus, the dentate gyrus, and substantia nigra *(8)*. This had led to speculation that the GHS receptor (GHS-R), and therefore its endogenous ligand, may play a role in processes that are not generally associated with the control of GH release.

The cloning of the GHS-R has provided a context in which agonist/receptor interactions can be investigated systematically. This has revealed that several structurally diverse compounds are capable of binding to the receptor and that different classes of GHSs can bind to different regions of the ligand-binding pocket of the receptor *(9)*. This, in turn, has led to the generation of several novel GHSs that may activate intracellular pathways different from those by conventional GHSs, possibly leading to a broader range of physiologic actions.

Recent studies have demonstrated that some GHSs are capable of eliciting the release of vasopressin from hypothalamic explants *(10)*, and vasopressin release is thought to, at least partially, underlie the hypothalamo-pituitary-adrenal axis activation induced by GHSs *(11)*. Cell bodies of vasopressin neurons are located in the SON, SCN, and paraventricular nucleus (PVN) of the hypothalamus *(12)*, and all these nuclei express GHS-R mRNA *(8)*. However, previous studies have shown that GH-releasing peptide-6 (GHRP-6) and MK-0677 do not elicit Fos immunoreactivity within these regions *(3,4)*. As within the hypothalamic-neurohypophyseal system, the use of Fos immunocytochemistry as a marker of neuronal activity is considered a reliable and intensity-dependent stimulus *(13)*; it appears that GHRP-6 and MK-0677 do not activate cells in the SON or PVN. However, because novel GHSs may interact with the GHS-R in a different manner from GHRP-6 or MK-0677, the apparent disparities between these studies may reflect activation of different regions of the GHS-R.

Therefore, in the present study, we employed the immunocytochemical detection of Fos protein in the rat to com-

Author to whom all correspondence and reprint requests should be addressed: Alex Bailey, Eurosterare Administrator, Centre for Reproductive Biology, Edinburgh EH3 9ET. E-mail: alex.bailey@obg93.obg.ed.ac.uk

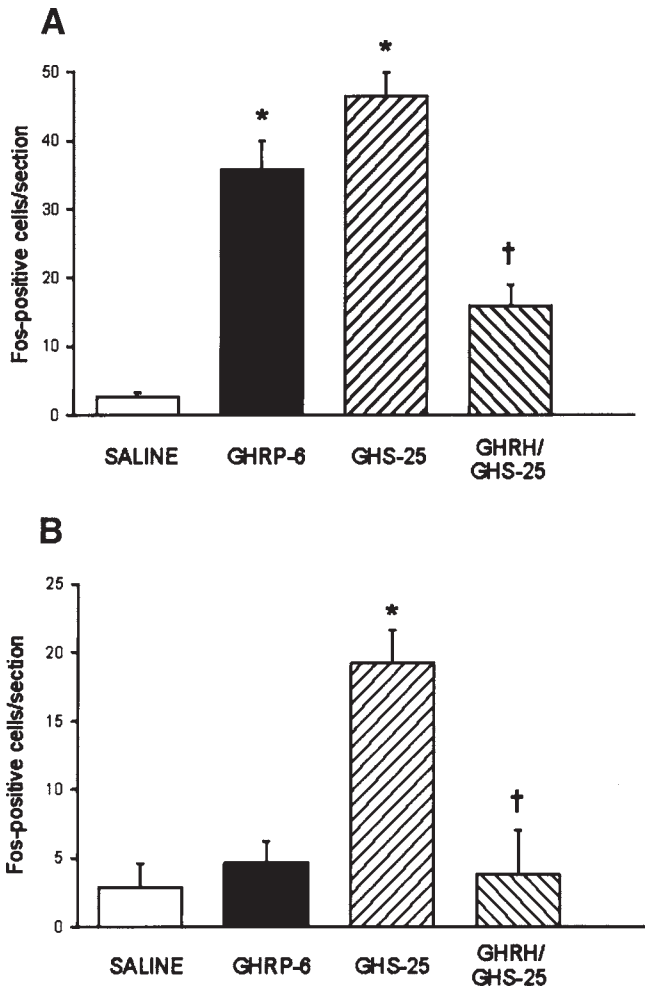

Fig. 1. Histogram showing the mean number of Fos-positive cells in **(A)** the arcuate nuclei and **(B)** the supraoptic nuclei of rats after iv injection of saline (0.2 mL; $n = 7$), GHRP-6 (50 µg; $n = 6$), GHS-25 (50 µg; $n = 8$), GHRH (2 µg)/GHS-25 (50 µg; $n = 6$). $^{*}p < 0.05$ compared with saline-treated animals; $^{\dagger}p < 0.05$ compared with GHS-25-treated animals.

pare the hypothalamic actions of GHRP-6 and a novel nonpeptidyl GHS, GHS-25. Additionally, we used double-labeling immunocytochemistry to identify the biochemical phenotype of hypothalamic cells activated by administration of these GHSs.

Results

Immunocytochemical Detection of Fos Protein

Systemic injection of GHRP-6 resulted in a significant increase in arcuate nucleus Fos immunoreactivity in all animals compared with saline-treated controls (Fig. 1). Intravenous injection of GHS-25 also resulted in significant increases in arcuate nucleus Fos protein expression (46.4 (3.5 Fos-positive nuclei per section; $p < 0.05$ compared with saline-treated controls). Prior injection of GHRH significantly reduced the amount of GHS-induced Fos immunoreactivity in the arcuate nucleus to 15.9 ± 3.0 Fos-positive nuclei per section ($p < 0.05$; Fig. 1).

The amount of Fos immunoreactivity in the SON of animals injected with GHRP-6 was not significantly different from that of saline-treated animals (2.9 ± 1.7 and 4.7 ± 1.5 Fos-positive nuclei per section, respectively). Following injection of GHS-25, there was a significant increase in the number of Fos-positive cells in the SON of all rats compared with GHRP-6-treated animals (19.2 ± 2.4 Fos-positive nuclei per section; $p < 0.05$) (Fig. 1). Although distributed throughout the rostrocaudal extent of the SON, Fos immunoreactivity was generally found within the dorsal regions of the nucleus (Fig. 2). Given alone GHRH did not induce any appreciable amount of Fos immunoreactivity in the SON, but injection of GHRH prior to GHS-25 significantly reduced the amount of SON Fos immunoreactivity to 3.8 ± 3.2 Fos-positive nuclei per section ($p < 0.05$, Fig. 1). GHS-25 did not induce appreciable levels of Fos protein expression within either the magnocellular or parvocellular regions of the PVN or elsewhere in the hypothalamus.

Immunocytochemical Double Labeling for Fos and Vasopressin or Oxytocin

In five rats, there were no significant differences in the total amount of Fos immunoreactivity in the SON of sections that had undergone double labeling for oxytocin (21.5 ± 1.9 Fos-positive nuclei per section) or vasopressin (18.4 ± 0.7 Fos-positive nuclei per section). Furthermore, neither of these values was significantly different from the amount of Fos protein present in the SON of animals that were only processed for the immunocytochemical detection of Fos. Double labeling revealed that 2.7 ± 0.3 cells/section were immunoreactive for both Fos and vasopressin, whereas significantly more cells (14.2 ± 1.5 cells/section; $p < 0.05$) were immunoreactive for both Fos and oxytocin (Fig. 2). Thus, approx 66% of all Fos-positive cells in the SON of animals injected with GHS-25 also contained oxytocin and approx 15% contained vasopressin. An additional 19% of Fos-positive cells could not be conclusively identified as immunoreactive for either oxytocin or vasopressin.

Discussion

Although previous studies have indicated that GHSs can alter the release of neurohypophyseal hormones in vitro *(10)*, this is the first study to show that GHSs can act on the SON in vivo. Mapping studies have demonstrated the existence of GHS-R mRNA within the SON *(8)*, although this and previous studies failed to show any induction of Fos protein in the SON following central or systemic administration of MK-0677 or GHRP-6 *(3,4)*. This is consistent with the observations that some nonpeptide GHSs bind to a different receptor domain than MK-0677 and GHRP-6 *(9)* and that binding in this region of the receptor induces the c-*fos* gene. Although relatively high levels of GHS-R mRNA have been observed throughout the central nervous system *(14)*, we did not observe induction of Fos protein in any

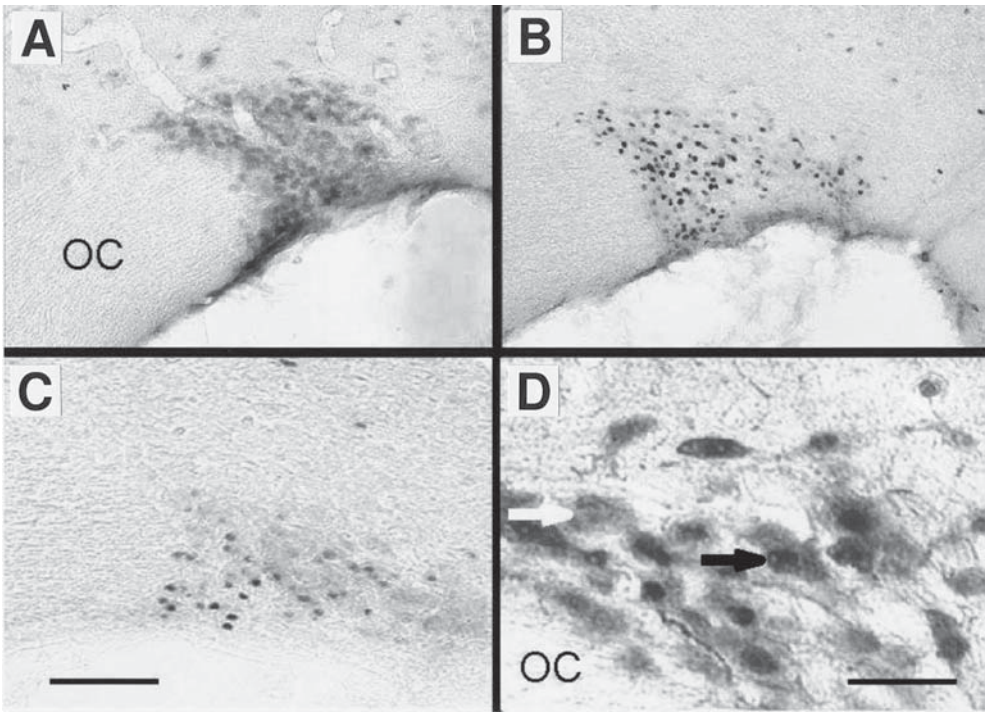

Fig. 2. Photomicrographs of the supraoptic nuclei of male rats showing the distribution of Fos protein immunoreactivity after iv injection of **(A)** GHRP-6 (50 µg), **(B)** GHS-25 (50 µg), and **(C)** GHRH (2 µg)/GHS-25 (50 µg). Scale bar = 0.2 mm. **(D)** Photomicrograph of the SON showing immunocytochemical double labeling for Fos and oxytocin. The white arrow points to a cell that is cytoplasmically labeled for oxytocin alone and the black arrow points to a cell that is double labeled, i.e., cytoplasmically labeled for oxytocin and nuclearly labeled for Fos. Scale bar = 100 µm. OC, optic chiasm.

region of the forebrain of GHS-25-injected rats, apart from the arcuate nucleus and SON. This observation does not preclude activation of neurons in regions expressing GHS-R mRNA because neuronal activation in these regions may not involve the induction of the c-*fos* gene. Administration of GHS-25 consistently induced Fos protein expression largely in the dorsal region of this nucleus, which is generally considered to contain the highest concentration of oxytocin neurons *(15)*. Double labeling confirmed that the majority of Fos-positive cells (approx 66%) also contained oxytocin. Clearly, Fos immunoreactivity was not induced in all SON cells, or all SON oxytocin cells, and the amount of induction (approx 20 cells/section) approximates that seen after a single systemic injection of choleocystokinin *(16)*.

In various species, it has been reported that GHSs do not influence plasma levels of neurohypophyseal hormones *(17,18)*. However, in hypothalamic explants and in humans, GHSs have been shown to increase vasopressin release *(10,19)*, indicating interactions between GHS and neurohypophyseal hormones. There are no reports of GHSs affecting plasma oxytocin levels, although this may depend on the structure of the GHSs in question. Induction of Fos protein in SON oxytocin cells does not necessarily correlate with increased oxytocin release *(20)*, and direct measurements of plasma oxytocin concentrations are needed to confirm the stimulatory effects of GHS-25.

The presence of GHS-R mRNA in the SON and activation of SON neurons by GHS-25 imply that the endogenous ligand may play a role in neurohypophyseal hormone release, although the physiologic significance of such a role is unclear. Neurohypophyseal hormones can alter GH release as indicated by decreased GH levels following immunoneutralization of oxytocin or vasopressin , and oxytocin has dose-dependent effects on plasma GH levels in vivo *(22)*. Therefore, within the SON, the endogenous ligand to the GHS-R may be indirectly involved in regulating GH release.

In the arcuate nucleus, GHS-25-induced Fos protein expression was attenuated by prior administration of GHRH, as previously reported for other GHSs *(23)*. Similarly, the administration of GHRH also reduced GHS-25-induced activation of the SON. Because GHRH is not an antagonist of the GHS-R, the reduction in SON Fos expression probably reflects a secondary action of GH release. This could include an inhibitory action following direct activation of GH receptors, which are present in the SON *(24)*, or a more indirect feedback pathway. GHS-induced activation of the arcuate nucleus can be attenuated by prior administration of a somatostatin analog *(25)*, and in mice, the negative feedback effects of GH are thought to be mediated by somatostatin receptor subtype 2 *(26)*. Thus, although a direct effect of GH within the SON cannot be excluded, the inhibitory actions of GHRH in the SON are more likely to be mediated by an increase in hypothalamic somatostatinergic tone.

In summary, GHS-25, in common with other GHSs, exerts actions within the arcuate nucleus indicative of the GHS-R's involvement in the regulation of GH release. Additionally, GHS-25 activates oxytocin cells in the SON, suggesting that the GHS-R may also play a role in controlling neurohypophyseal hormone release.

Materials and Methods

Animals and Drugs

Adult male Sprague-Dawley rats (250–300 g) were maintained in a controlled environment (14-h light, 10-h dark cycle; 21–22°C) with water and food available ad libitum. GHS-25 was obtained from Merck. GHP-6 was obtained from Peninsula and GHH from Bachem. All drugs were dissolved in 0.2 mL of isotonic saline before administration.

Immunocytochemical Detection of Fos Protein

Rats were briefly anesthetized with halothane (5% halothane in an O_2/NO_2 mixture) and a polythene cannula was secured in the right jugular vein for iv drug administration. After 24 h of recovery, the rats were injected with saline (0.2 mL), GHP-6 (50 μg), or GHS-25 (50 μg). In an additional study to examine the effects of endogenous GH on the induction of Fos in the hypothalamus, a number of animals were injected intravenously with GHH (2 μg) 10 min prior to iv injection of GHS-25 (50 μg).

Ninety minutes after injection, rats were terminally anesthetized with sodium pentobarbitone (60 mg/kg intravenously) and perfused transcardially with heparinized saline followed by 4% paraformaldehyde (in 0.1 *M* phosphate buffer [PB]). Whole brains were then removed and placed in 30% sucrose (in 4% paraformaldehyde) for 24 h before being frozen on dry ice and stored at –80°C until processing. Coronal sections (every second section; 50 μm thickness) were cut on a freezing microtome and placed in 0.1 *M* PB containing Triton X-100 ([PB-T], pH 7.4). Endogenous peroxidases were deactivated in a solution of PB-T containing 20% methanol and 0.3% H_20_2 for 15 min. Sections were incubated for 36 h at 4°C in Ab-2 Fos antibody (rabbit polyclonal, 1:1000 in 1% normal sheep serum; Oncogene Science). The antibody-antigen complex was localized with a 1-h incubation in biotinylated antirabbit immunoglobulin (1:1000) (Vector), followed by a 1-h incubation in avidin-biotinylated horseradish peroxidase (1:50) (Vector). The reaction product was visualized using a glucose oxidase-DAB-nickel method producing a purple-black stain restricted to the nucleus.

Immunocytochemical Double Labeling for Fos and Vasopressin or Oxytocin

To identify the biochemical phenotype of neurons displaying Fos immunoreactivity following the administration of GHSs, a number of animals were surgically prepared as already described and after 24 h of recovery injected with GHS-25 (50 μg intravenously). After 90 min, rats were terminally anesthetized with sodium pentobarbitone (60 mg/kg intravenously) and brains were processed for the immunocytochemical detection of Fos protein as already described.

Hypothalamic sections were then divided into thee groups, two of which underwent labeling for either oxytocin or vasopressin. Briefly, sections were washed in 0.1 *M* PB and incubated at 4(C in primary antibody of either oxytocin or vasopressin (both 1:2000 in 1% normal sheep serum). The following day, sections were rinsed in 0.1 *M* PB and the antibody-antigen complex was localized with a 1-h incubation in biotinylated antirabbit immunoglobulin (1:100) (Vector), followed by a 1-h incubation in avidin-biotinylated horseradish peroxidase (1:50) (Vector). Visualization was achieved using a DAB-peroxide method producing a brown-red stain restricted to the cytoplasm.

For each rat, the number of Fos-positive cells in the respective hypothalamic nuclei (6–10 sections/rat) was counted bilaterally and a mean calculated for each experimental group (mean ± SEM nuclei/section). For the double-labeling immunocytochemistry, the number of SON Fos-positive cells that also contained cytoplasmic staining for oxytocin or vasopressin was counted for each section (six to seven sections per rat). Statistical analysis was performed by a one-way analysis of variance followed by a student-Newman-Keuls post-hoc test with significance taken as $p < 0.05$. All data are expressed as mean ± SEM.

Acknowledgments

We wish to thank Michelle Giles for her assistance with the double-labeling immunocytochemistry and Ravi Nargund and Jim Tata for supplying the GHSs. This work was supported by Merck Research Laboratories, and The Medical Research Council, UK.

References

1. Bowers, C. Y., Momany, F. A., Reynolds, G. A., and Hong, A. (1984). *Endocrinology* **114,** 1537–1545.
2. Smith, R. G., Pong, S. S., Hickey, G., et al. (1996). *Recent Prog. Horm. Res.* **51,** 261–285.
3. Dickson, S. L., Leng, G., and Robinson, I. C. (1993). *Neuroscience* **53,** 303–306.
4. Bailey, A. R., Smith, R. G., and Leng, G. (1998). *J. Neuroendocrinol.* **10,** 111–118.
5. Dickson, S. L. and Luckman, S. M. (1997). *Endocrinology* **138,** 771–777.
6. Tannenbaum, G. S., Lapointe, M., Beaudet, A., and Howard, A. D. (1998). *Endocrinology* **139,** 4420–4423.
7. Willesen, M. G., Kristensen, P., and Mer, J. (1999). *Neuro-Endocrinology* **70,** 306–316.
8. Guan, X. M., Yu, H., Palyha, O. C., McKee, K. K., Feighner, S. D., Sirinathsinghji, D. J., Smith, R. G., Van der Ploeg, L. H., and Howard, A. D. (1997). *Brain Res. Mol. Brain Res.* **48,** 23–29.
9. Feighner, S. D., Howard, A. D., Prendergast, K., et al. (1998). *Mol. Endocrinol.* **12,** 137–145.
10. Korbonits, M., Little, J. A., Forsling, M. L., Tringali, G., Costa, A., Navarra, P., Trainer, P. J., and Grossman, A. B. (1999). *J. Neuroendocrinol.* **11,** 521–528.

11. Korbonits, M., Kaltsas, G., Perry, L. A., Putignano, P., Grossman, A. B., Besser, G. M., and Trainer, P. J. (1999). *J. Clin. Endocrinol. Metab.* **84,** 2489–2495.
12. Dierickx, K. (1980). *Int. Rev. Cytol.* **62,** 119–185.
13. Hoffman, G. E., Smith, M. S., and Verbalis, J. G. (1993). *Front Neuroendocrinol.* **14,** 173–213.
14. Bennett, P. A., Thomas, G. B., Howard, A. D., Feighner, S. D., Van der Ploeg, L. H., Smith, R. G., and Robinson, I. C. (1997). *Endocrinology* **138,** 4552–4557.
15. Armstrong, W. E. (1995). *Prog. Neurobiol.* **47,** 291–339.
16. Luckman, S. M., Hamamura, M., Antonijevic, I., Dye, S., and Leng, G. (1993). *Br. J. Pharmacol.* **110,** 378–384.
17. Arvat, E., Maccagno, B., Ramunni, J., Di Vito, L., Broglio, F., Deghenghi, R., Camanni, F., and Ghigo, E. (1997). *Neuro-Endocrinology* **66,** 432–438.
18. Hickey, G. J., Jacks, T. M., Schleim, K. D., Frazier, E., Chen, H. Y., Krupa, D., Feeney, W., Nargund, R. P., Patchett, A. A., and Smith, R. G. (1997). *J. Endocrinol.* **152,** 183–192.
19. Rosen, R. C., Lane, R. M., and Menza, M. (1999). *J. Clin. Psychopharmacol.* **19,** 67–85.
20. Luckman, S. M., Dyball, R. E., and Leng, G. (1994). *J. Neurosci.* **14,** 4825–4830.
21. Franci, C. R., Anselmo-Franci, J. A., Kozlowski, G. P., and McCann, S. M. (1993). *NeuroEndocrinology* **57,** 693–699.
22. Bjorkstrand, E., Hulting, A. L., and Uvnas-Moberg, K. (1997). *Regul. Pept.* **69,** 1–5.
23. Bailey, A. R., Honda, K., Smith, R. G., and Leng, G. (1999). *NeuroEndocrinology* **70,** 101–106.
24. Minami, S., Kamegai, J., Hasegawa, O., Sugihara, H., Okada, K., and Wakabayashi, I. (1993). *J. Neuroendocrinol.* **5,** 691–696.
25. Dickson, S. L., Viltart, O., Bailey, A. R., and Leng, G. (1997). *NeuroEndocrinology* **66,** 188–194.
26. Zheng, H., Bailey, A., Jiang, M. H., Honda, K., Chen, H. Y., Trumbauer, M. E., Van der Ploeg, L. H., Schaeffer, J. M., Leng, G., and Smith, R. G. (1997). *Mol. Endocrinol.* **11,** 1709–1717.