

Role of substance P in suppressing growth hormone release in the rat

(third ventricular and intravenous injection/dispersed anterior pituitary cells/substance P antiserum/substance P antagonist/plasma and pituitary incubation medium growth hormone)

M. ARISAWA*, G. D. SNYDER†, L. DE PALATIS‡, R. H. HO§, R. K. XU¶, G. PAN||, AND S. M. MCCANN**,††

*Department of Obstetrics/Gynecology, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160, Japan; †Department of Obstetrics/Gynecology, University of Iowa, Building MRF, Room 466, Iowa City, IA 52242; ‡The Dow Chemical Company, M. E. Pruitt Research Center, 1701 Building, Midland, MI 48674; §Department of Anatomy, The Ohio State University, 4072 Graves Hall, 333 West 10th Avenue, Columbus, OH 43210-1239; ¶Department of Physiology, Institute of Basic Medical Science, Chinese Academy of Medical Science, 5 Dong Dan San Tiao, Beijing, China; ||Department of Physiology and Biophysics, University of Alabama in Birmingham, Birmingham, AL 35294; and **Department of Physiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235

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ABSTRACT To evaluate a possible physiological role of endogenous substance P (SP) in the control of growth hormone (GH; somatotropin) secretion, a specific antiserum against SP (anti-SP) was injected intraventricularly (3 μ l into the third cerebral ventricle) in unanesthetized unrestrained normal male rats. Control rats received an equivalent volume of normal rabbit serum (NRS). Intraventricular injection of the NRS lowered plasma GH concentrations significantly. The lowering was detected on first measurement at 10 min after injection and was maximal at 30 min. This was followed by a return toward the initial levels. Third ventricular injection of antiserum significantly increased plasma GH in comparison with control animals injected with NRS. The effect was observed within 10–20 min, and levels remained elevated for the 120-min duration of the experiment. To confirm the possible inhibitory role of endogenous SP on GH release, 3 μ l of 0.9% NaCl (saline) alone or saline containing a specific antagonist of SP, [D-Pro²,D-Trp^{7,9}]SP, was injected into the third ventricle of normal male rats. The antagonist also increased plasma GH significantly ($P < 0.005$) within 5 min compared with values in the saline-injected control group. Levels remained elevated for 30 min but had returned toward control values 60 min after injection. In contrast, synthetic SP significantly decreased plasma GH when injected intravenously or intraventricularly compared with plasma GH in the control saline-injected group. To investigate a possible direct action of SP on GH release from the anterior pituitary gland, we incubated synthetic SP with dispersed anterior pituitary cells for 1 hr. The release of GH from incubated anterior pituitary cells was not affected at any dose of SP (10^{-9} to 10^{-6} M) tested. These data strongly indicate that endogenous SP has a physiological inhibitory role in the control of GH secretion at the level of the hypothalamus in the male rat.

Substance P (SP) was originally detected in equine brain and intestine by Von Euler and Gaddum in 1931 (1). This undecapeptide was isolated from the hypothalamus and characterized by Chang and Leeman in 1970–1971 (2, 3). Immunocytochemical studies have demonstrated an SP-containing neuronal system in the hypothalamus (4–10), and the peptide has been localized to cells of the anterior pituitary gland as well (11, 12). In addition, SP receptors have been localized to various regions of the hypothalamus and pituitary (5, 6). These findings suggest that SP may have a role in the control of pituitary hormone secretion.

Growth hormone (GH; somatotropin) release from the pituitary is under dual control by a stimulating factor, GH-releasing hormone (GHRH; somatoliberin), and an inhibitory peptide, GH release-inhibiting hormone (somatostatin). These peptides are released into the hypophyseal portal vessels and are carried to the pituitary, where they stimulate or inhibit the release of GH from the somatotrophs. Both inhibitory and stimulatory effects of SP on GH release have been reported, so the role of this peptide in control of GH secretion is controversial (4). Consequently, in the present study we reevaluated the role of SP in the control of GH release in the male rat by injecting it into the third cerebral ventricle (3V) and by also incubating it with dispersed anterior pituitary cells *in vitro*. To determine the physiological role of endogenous SP in secretion of GH, we have employed a specific antiserum against SP and also an antagonist of the peptide, [D-Pro²,D-Trp^{7,9}]SP.

MATERIALS AND METHODS

Materials. The experiments employed male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing 250–300 g. Rats were maintained under controlled lighting (lights on 05:00–19:00) and temperature ($24 \pm 1^\circ\text{C}$). Rat chow and water were available ad lib. The antiserum used (R-11) has been fully characterized (11) and is directed at the carboxyl terminus. Peptides structurally related to SP had either negligible or no detectable immunoreactivity with the antiserum. Other peptides tested that exhibited no detectable crossreactivity with the antiserum were luteinizing hormone-releasing hormone, neurotensin, cholecystokinin, [arginine]vasotocin, [methionine]enkephalin, adrenocorticotropin, vasoactive intestinal polypeptide, and bradykinin. Synthetic SP and [D-Pro²,D-Trp^{7,9}]SP were obtained from Peninsula Laboratories.

In Vivo Experiments. Experiment I. Six to 8 days before the experiment, a 23-gauge stainless steel cannula (17 mm in length) was implanted into the 3V as described earlier (13). Each cannula was provided with a mandril to prevent its obstruction. The location of the cannula in the 3V was confirmed when cerebrospinal fluid flowed continuously from the cannula. After implantation, the animals were placed in individual cages until the day of the experiment. Twenty-four hours prior to the experiment, an indwelling catheter was inserted into the right jugular vein by using the technique of Harms and Ojeda (14) while the animal was

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Abbreviations: SP, substance P; GH, growth hormone; GHRH, GH-releasing hormone; 3V, third cerebral ventricle; NRS, normal rabbit serum.

††To whom reprint requests should be addressed.

anesthetized with ether. On the morning of the experiment, the animal was left undisturbed for at least 60 min. Three microliters of undiluted antiserum or an equal volume of normal rabbit serum (NRS) was administered immediately after withdrawal of the first blood sample (0.8 ml). Blood samples (0.8 ml) were withdrawn at various time intervals thereafter. In this and subsequent experiments, each blood sample was replaced with an equal volume (0.8 ml) of saline (0.9% NaCl).

Experiment II. In rats with indwelling 3V cannulae 3 μ l of the undiluted antiserum or an equal volume of NRS was injected intraventricularly 24 hr before the initial blood sampling and just prior to implantation of a catheter in the external jugular vein. On the day of the experiment, one blood sample was withdrawn and the animal was injected intraventricularly with 3 μ l of the undiluted antiserum or NRS. Blood samples were withdrawn at various time intervals thereafter as described above.

Experiment III. In rats with implanted ventricular and jugular cannulae, [D-Pro²,D-Trp^{7,9}]SP (50 ng in 3 μ l of saline) or an equal volume of saline was injected into the 3V immediately after the initial blood sampling. Blood samples were taken thereafter as described above.

Experiment IV. To evaluate the effect of SP itself, synthetic SP dissolved in 3 μ l of 0.9% NaCl, or an equal volume of saline alone, was injected into the 3V in rats implanted with ventricular and jugular cannulae immediately after the first blood sample (0.8 ml) was withdrawn. Blood samples (0.8 ml) were withdrawn just prior to intraventricular injections and at various time intervals thereafter.

Experiment V. In rats bearing only jugular catheters, an initial blood sample was withdrawn and various doses of SP were injected intravenously (i.v.). Blood samples were withdrawn at various intervals thereafter.

In Vitro Experiments. Anterior pituitaries were removed from adult male rats after decapitation and dispersed in the presence of 0.1% trypsin as previously described (15). Cells were incubated overnight at 37°C in medium 199 containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (GIBCO), 10% horse serum, and penicillin-streptomycin (1 ml/100 ml; GIBCO). On the day of experimentation, overnight culture medium was removed. The cells were incubated for 30 min in the above medium without horse serum but containing 0.1% bovine serum albumin (GIBCO). Then they were resuspended in either 1 ml of this medium alone or 1 ml of the same medium containing appropriate test substances. The incubation was terminated after 60 min. After centrifugation media were stored frozen at -20°C for subsequent analyses by radioimmunoassay.

Hormone Assays. GH was measured by specific radioimmunoassay using a kit supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). NIDDK-rat-GH-RP-1 was used as the reference preparation for each assay.

Statistical Analysis. The significance of the changes between pre- and postinjection levels of GH was calculated by using one-way analysis of variance and the Student-Newman-Keul multiple comparison test for unequal replication. Data from two different treatment groups at each time point and the difference between the areas under the curve of plasma GH were determined by Student's *t* test.

RESULTS

Effect of Intraventricular Injection of Antiserum to SP. Intraventricular injection of NRS (3 μ l into the 3V) resulted in a decrease in plasma GH which was significant on first sampling, 20 min after injection. Values reached a nadir at 40 min and gradually returned to initial levels by 240 min. A single injection of anti-SP into the 3V significantly increased

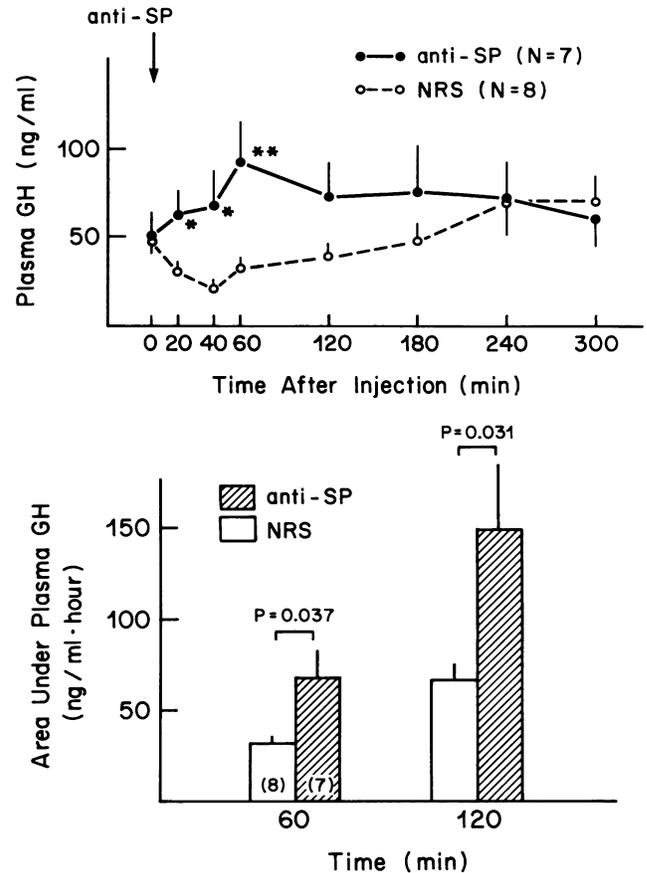


FIG. 1. (Upper) Effect of a single intraventricular injection (arrow) of anti-SP antiserum (3 μ l into the 3V) or an equal volume of NRS on plasma GH levels. In this and subsequent figures, the values obtained are given as the mean \pm SEM and *N* is the number of samples. *, $P < 0.05$; **, $P < 0.025$ vs. control (NRS) at the same time. (Lower) Area under the plasma GH curve in Upper at two times.

plasma GH as compared to control animals injected with NRS (Fig. 1 Upper). The effect was observed within 20 min after administration and plasma GH remained elevated for 180 min. Fig. 1 Lower depicts the area under the curve of plasma GH in both groups for the first hour and the initial 2 hr after injection. The area under the plasma GH curve of the anti-SP-injected group was increased more than 2-fold as compared to that of the control group at both time points.

To confirm the stimulatory effect of anti-SP on GH secretion, anti-SP or NRS was injected into the 3V 24 hr prior to the experiment. The injections were repeated just after removal of the first blood sample. This double-injection protocol was followed since it had been found effective in previous passive immunization experiments (16). Although plasma GH was not significantly altered 24 hr after the injection in comparison with the values in the NRS-injected control groups, there was a greater effect of the second intraventricular injection of antiserum than that seen after a single injection. Plasma GH was already significantly higher within 10 min after the second injection compared to the decreased levels of control animals which received two injections of NRS (Fig. 2 Upper). The difference between groups was maintained for the 90-min duration of the experiment. When the area under the curve of plasma GH was determined on both groups, the area was significantly greater ($P < 0.01$) for the anti-SP-injected group than for the control group (Fig. 2 Lower).

Effect of Intraventricular Injection of the SP Antagonist. [D-Pro²,D-Trp^{7,9}]SP caused a significant increase of plasma

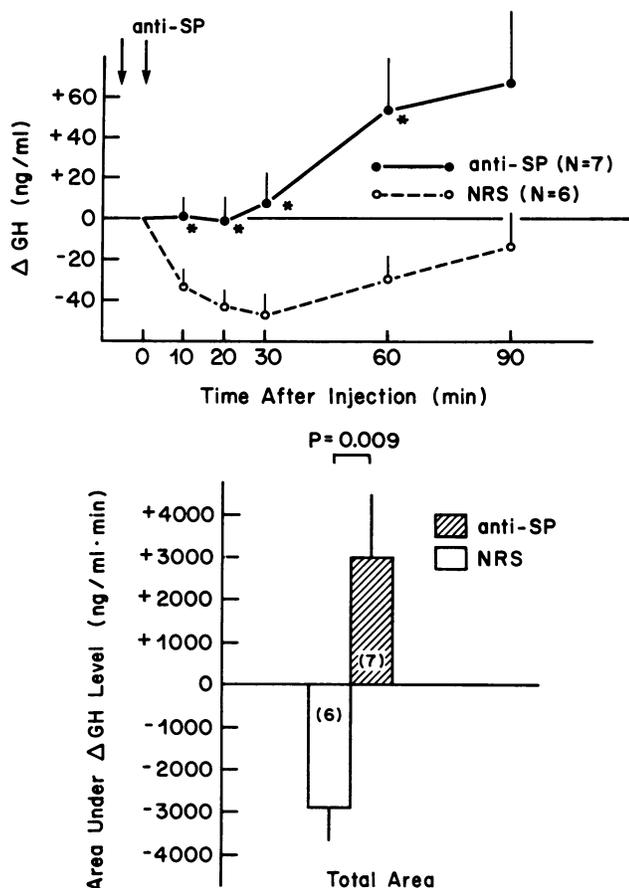


FIG. 2. (Upper) Effect of two intraventricular injections (arrows) of anti-SP ($3 \mu\text{l}$) or NRS on GH release. The first injection into the 3V occurred 24 hr before the experiment. A second was given immediately after the first blood sampling. Because of variation between groups in initial values, for greater clarity results are presented as change in plasma GH (Δ GH) at various times after injection. *, $P < 0.025$ vs. NRS at the same time. The initial values prior to the second injection were 70.8 ± 10.9 ng/ml for NRS-injected rats and 52.3 ± 9.3 ng/ml for those injected with anti-SP serum. (Lower) Areas under the plasma Δ GH curves.

GH within 5 min of its injection into the 3V, and the levels remained elevated for 30 min after injection compared with levels in the saline-injected control group ($P < 0.01$) (Fig. 3 Upper). By 60 min after injection, values had returned toward control levels. The area under the plasma GH curves of both groups revealed a highly significant increase ($P < 0.005$) in area in the antagonist-injected rats above that of diluent-injected animals (Fig. 3 Lower).

Effect of Intraventricular Injection of SP. Intraventricularly administered SP [$1 \mu\text{g}$ (0.7 nmol) or $0.1 \mu\text{g}$ (0.07 nmol)] significantly suppressed GH release in comparison with the levels in saline-injected control animals, as indicated by a lowering of plasma GH (Fig. 4). The plasma GH decreased slightly but not significantly more after the higher dose and reached a nadir at 40 min after injection.

Effect of Intravenous Injection of SP. Systemic injection of 1-, 10-, and 50- μg doses of SP induced a significant decrease in plasma GH, as shown in Fig. 5. The effect was observed within 10 min after injection, and levels remained suppressed for 60 min compared with the concentrations in the saline-injected control group. The suppression of plasma GH was dose dependent. The suppressive effects of the 10- or 50- μg doses of SP were nearly equal at 40 and 60 min and were significantly greater at 40 min than those of the 1- μg dose.

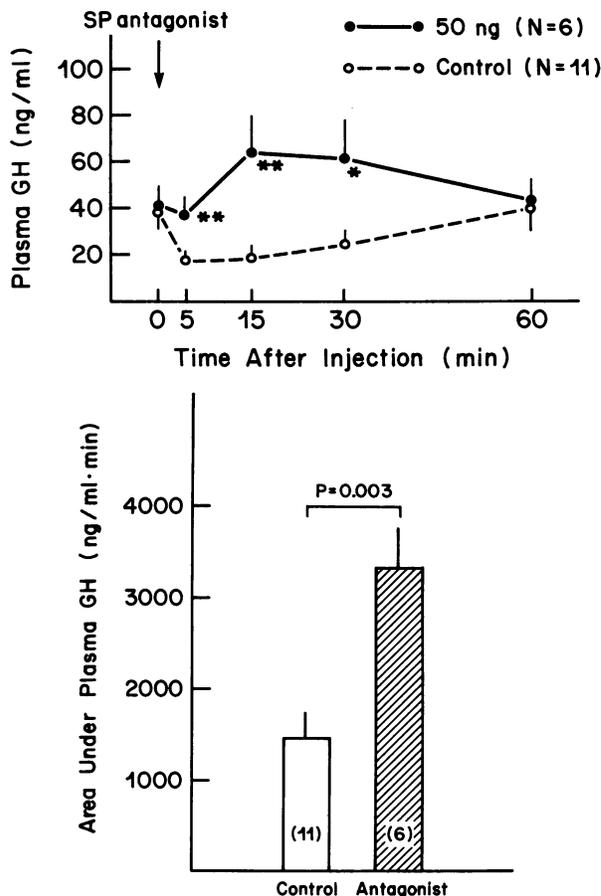


FIG. 3. (Upper) Effect of intraventricular injection (arrow) of [D -Pro 2 , D -Trp 7,9]SP (50 ng) or physiological saline on GH release. *, $P < 0.025$; **, $P < 0.01$ vs. control at the same time. (Lower) Areas under the plasma GH curves.

In Vitro Studies. When primary cultures of anterior pituitary cells from normal male rats were incubated *in vitro* with SP, none of the concentrations (10^{-9} to 10^{-6} M), had any

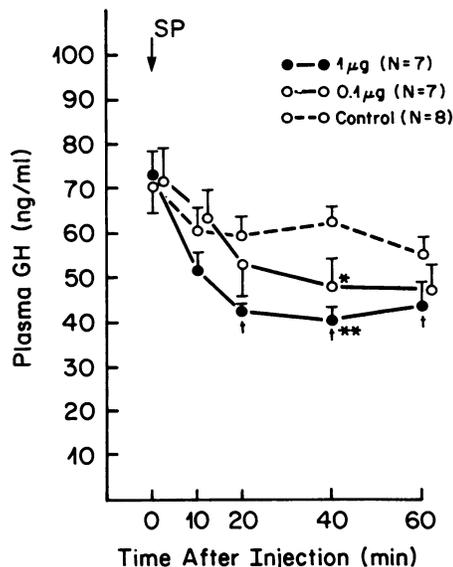


FIG. 4. Effect of intraventricularly injected (arrow) SP or physiological saline on GH release in unanesthetized normal male rats. *, $P < 0.05$; **, $P < 0.025$ vs. control value at the same time. †, $P < 0.001$ vs. zero-time sample.

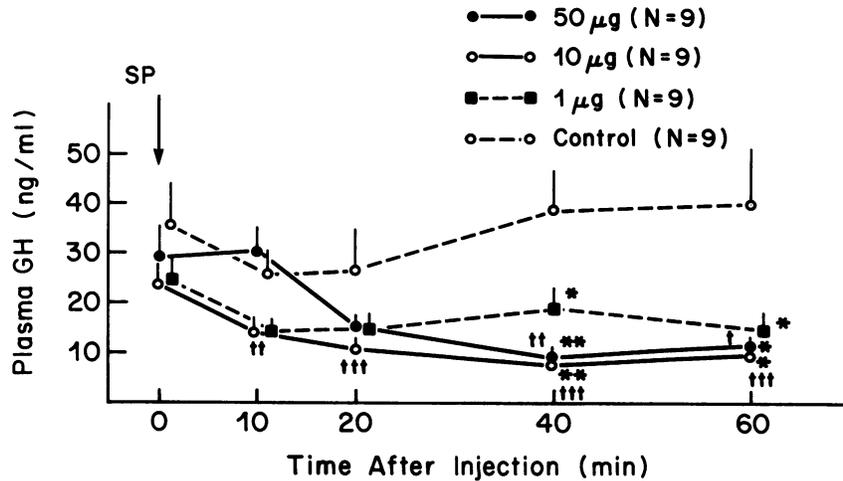


FIG. 5. Effect of i.v. injected (arrow) SP or physiological saline on GH release in unanesthetized normal male rats. *, $P < 0.005$; **, $P < 0.001$ vs. control value at the same time. †, $P < 0.025$; ††, $P < 0.01$; †††, $P < 0.001$ vs. zero-time sample.

significant effect on the release of GH into the culture medium, but the cells were responsive to GHRH (Table 1).

DISCUSSION

The present results indicate that both a specific antiserum against SP and a specific antagonist of the peptide can cause an increase of plasma GH when injected into the 3V of male rats. In contrast, exogenous SP suppressed the release of GH when administered either intravenously or intraventricularly. The minimal effective dose after 3V injection was less than that after i.v. injection, which indicates a central inhibitory action of SP. These data strongly suggest that endogenous SP has a physiological inhibitory role in the control of GH secretion at the hypothalamic level in normal male rats. In most instances the control injections of either saline or NRS suppressed plasma GH in these animals, which was probably related to a stress-induced inhibition of GH release (17). Therefore, part of the action of the antiserum or the antagonist may be related to the blockade of stress-induced somatostatin release; however, since the antagonist elevated plasma GH to levels clearly above normal, it would appear that GHRH release was stimulated as well.

To date, both inhibitory and stimulatory effects of SP upon GH release have been reported, and the physiological role of this peptide in the control of GH secretion is still controversial (4–6). It was reported that systemic administration of SP stimulated GH release in urethane-anesthetized male rats (18, 19). However, in the present study, we were unable to stimulate GH secretion with 1, 10, or 50 µg of SP in conscious, freely moving normal male rats. To confirm the effect of i.v. SP on GH release, we injected the same amounts of SP (1, 10, and 50 µg per animal) as used here into ovariectomized unanesthetized rats and unanesthetized estrogen-progesterone-primed ovariectomized rats. No stimu-

lation of GH release by SP was observed in either of these cases and a decreased plasma GH was observed as in males (data not shown). Therefore, we conclude that SP has an inhibitory effect on GH release when injected i.v. into unanesthetized rats. The discrepancy between our results and those previously reported may be related to the use of anesthetized instead of conscious rats, since anesthetics have been clearly shown to modify the response to injected drugs in some situations (20). 3V injection of SP into ovariectomized, conscious female rats was reported to cause an increase in plasma GH (21), whereas Chihara *et al.* (22), in agreement with the present results, reported the inhibitory effect of this peptide when injected into the lateral ventricle of urethane-anesthetized male rats. They postulated that SP may exert an inhibitory effect on GH secretion via hypothalamic release of somatostatin, since the effect of SP was blocked in animals previously injected with antiserum to somatostatin. Other investigators (23) reported increased *in vitro* release of somatostatin from hypothalamic fragments incubated in the presence of SP, which supports the concept that the central inhibitory effect of SP on GH may be mediated by somatostatin. On the other hand, Takahara *et al.* (24) found that intraventricularly injected SP itself had no effect on serum GH, but it inhibited the γ -aminobutyric acid-induced increases in serum GH. In the rhesus monkey, SP was also reported to induce a significant decrease in GH secretion (24).

It is difficult to assign a physiological role for SP in the control of GH secretion from injections of the peptide, since microgram quantities of SP might have some pharmacological effect on the central nervous system when injected intraventricularly, and systemic administration of SP could induce changes in blood pressure or other effects and provoke a stress response. Therefore, neutralization of endogenous SP by the use of an antiserum or an antagonist is an effective method to investigate the physiological role of SP. In this study, we demonstrated that synthetic SP inhibits GH release, whereas anti-SP and an antagonist to the peptide had the opposite effect, increasing plasma GH after administration into the 3V. In view of these findings and our *in vitro* results which showed no direct effect of SP on the release of GH from anterior pituitary cells, we conclude that, in unanesthetized freely moving male rats, SP exerts an inhibitory role in the control of basal release of GH at the level of the hypothalamus.

Table 1. Effect of SP and GHRH on GH release from dispersed anterior pituitary cells harvested from normal male rats

Treatment	GH released, ng
Control	726.0 ± 24.2
10 ⁻⁶ M SP	716.0 ± 25.0
10 ⁻⁷ M SP	676.0 ± 35.2
10 ⁻⁸ M SP	736.0 ± 39.0
10 ⁻⁹ M SP	748.0 ± 38.3
4 × 10 ⁻⁹ M GHRH	1149.0 ± 45.5*

There were 5.0×10^5 cells per tube. Results are mean ± SEM for five samples.

* $P < 0.001$ vs. control.

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