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Growth Hormone-Releasing Hexapeptide Is a Potent Stimulator of Growth Hormone Gene Expression and Release in the Growth Hormone–Releasing Hormone–Deprived Infant Rat

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ABSTRACT

The growth hormone-releasing hexapeptide (GHRP-6) specifically stimulates growth hormone (GH) secretion in several animal species and humans. The mechanism of action of GHRP-6 is largely unknown, although experimental evidence indicates that it may modulate growth hormone–releasing hormone (GHRH) and somatostatin actions at the pituitary or hypothalamic level. To gain more insight into the mechanism(s) of action of GHRP-6, we studied the infant rat, an animal model highly responsive to GH-releasing stimuli. In 14-d-old rats GHRP-6 (32–600 μg/kg, s.c.) induced a marked and dose-dependent rise in plasma GH concentrations, maximal stimulation occurring with the dose of 300 μg/kg. Neither GHRH nor somatostatin antiserum prevented or modified the GH release elicited by GHRP-6. In pups passively immunized with GHRH antibodies, a 5-d treatment with GHRP-6 (80 μg/kg, s.c., twice daily) completely counteracted the inhibitory effect of GHRH deprivation on GH mRNA expression. *In vitro* GHRP-6 (10^{-7} and 10^{-6} M) induced a small and transient stimulation of GH release from cultured pituitary cells. These results indicate the following: *1)* GHRP-6 is a potent stimulator of GH release in rat pups; *2)* it stimulates GH gene expression in the GHRH-deprived pup; *3)* during the neonatal period its action is not mediated by GHRH or somatostatin; and *4)* its actions are not directed at the somatotrophs. (***Pediatr Res* 36: 169–174, 1994**)

Abbreviations

GHRP-6, growth hormone–releasing hexapeptide
GH, growth hormone
GHRH, growth hormone–releasing hormone
GHRH-ab, antiserum against growth hormone–releasing hormone
somatostatin-ab, antiserum against somatostatin
NRS, normal rabbit serum

GHRP-6 is a synthetic peptide that specifically stimulates growth hormone release in all species so far investigated (1–3). Although GHRP-6 has been shown to stimulate GH release *in vitro* from rat primary pituitary cell cultures (4, 5), it shows a far greater GH-releasing activity when administered *in vivo* (6–8). These observations suggest that GHRP-6 stimulates GH secretion by acting on two sites, the pituitary and the hypothalamus. In keeping with these findings, receptor binding sites for GHRP-6 have been demonstrated in both tissues (9). Experimental evidence has indicated that GHRP-6 does not bind to the GHRH receptor (7) or activate adenylate cyclase (4). However, *in vivo* studies have shown that GHRP-6 potentiates the GH-releasing effect of GHRH when the peptides are coadministered into humans (8) or adult rats (10), and passive immunization against GHRH attenuates the GH response to GHRP-6 (11). Overall, it appears that *in vitro* results imply independent mechanisms for GHRP-6 and GHRH at the level of the pituitary level (4, 6, 8), whereas *in vivo* findings suggest that GHRP-6 may modulate GHRH or somatostatin function at the hypothalamic level (9–11).

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To gain more insight into the mechanism(s) underlying the GH-releasing activity of GHRP-6, we used the infant rat, an animal model well suited for evaluating GH-releasing stimuli (12–14). First, we evaluated the GH-releasing effect of increasing doses of GHRP-6 *in vivo*. We then tested the GH-releasing effect of GHRP-6 *in vitro* and compared it to that occurring after GHRH. We then studied the involvement of GHRH and somatostatin in the mechanism of action of GHRP-6 by the use of passive immunization with specific antisera against GHRH and somatostatin. Finally, we evaluated the capacity of GHRP-6 to affect pituitary GH gene expression in rats deprived since birth of endogenous GHRH.

METHODS

Animals

Male and female 10- and 14-d-old Sprague-Dawley rats (Charles River Breeding Laboratories, Calco, Italy) weighing approximately 25 and 35 g, respectively, were used. They were received on the day of birth and were housed under controlled conditions (22 ± 2°C, 65% humidity and artificial light from 0600–2000 h). A standard dry diet and water were available *ad libitum* to the dams. One hour before the experiments, pups were separated from their respective dam and were randomly divided into groups of eight. All the experiments were performed in accordance with the Italian Guidelines for the Use of Animals in Medical Research.

Antiserum to GHRH and Somatostatin

The GHRH-ab, prepared and validated by one of us (W.B.W.), has been repeatedly found to inhibit normal GH secretion and slow growth (15–18). In a previous study (16) we evaluated the effect of GHRH-ab (50–200 μL/rat) injected into 10-d-old rats on GH secretion. On the basis of that study, a dose of 100 μL/rat was chosen because it maximally inhibited GH secretion. The somatostatin-ab was prepared by immunizing rabbits with synthetic somatostatin conjugated to BSA and glutaraldehyde. The antiserum is directed toward the middle part of somatostatin and does not cross-react with other hypothalamic, pituitary, and gastrointestinal peptide hormones. One hundred μL of the undiluted antiserum were able to neutralize approximately 600 ng of somatostatin, an amount that greatly exceeds that present in the 14-d-old rat.

Experimental Procedure

In vivo experiments. In the first series of experiments pups received a single s.c. injection of different doses of GHRP-6 (Bachem, Bubendorf, Switzerland) (*i.e.* 0, 16, 32, 150, 300, and 600 μg/kg dissolved in physiologic saline). Animals were killed by decapitation 20 min later.

In the second experiment, pups received a s.c. injection of 100 μL of a GHRH-ab (15) or somatostatin-ab (13) or 100 μL of both antiserum (GHRH-ab + somatostatin-ab), or 100 μL NRS. One h later they received a s.c. injection of GHRP-6 (300 μg/kg) and were killed by decapitation 20 min after GHRP-6 injection. Trunk blood was collected and immediately centrifuged. Serum samples were stored at −20°C until assayed for GH.

In the third experiment aimed at evaluating the ability of GHRP-6 to stimulate GH synthesis, pups were given GHRH-ab (100 μL/rat) on d 1, 2, 4, 6, 8, and 10 of age. Control-treated rats received an equal volume of NRS. Beginning on d 6 or 8 and up to d 10, pups were given GHRP-6 (80 μg/kg, s.c., twice daily) or physiologic saline. Twelve h after the last administration of GHRP-6, pups were killed, and the pituitary gland was quickly dissected and frozen on dry ice for the determination of GH gene expression.

In vitro experiments. Pups were killed by decapitation, and the pituitary glands were rapidly dissected. Pituitary tissue used for cell dissociation included both the anterior and posterior lobes. Briefly, pituitary glands were collected in sterile F-10 medium (Sigma Chemical Co., St. Louis, MO). Tissue was cut into small fragments and incubated twice for 15 min at 37°C in F-10 medium containing 6% FCS and collagenase (2.5 mg/mL) (Boehringer, Mannheim GmBH, Germany). Fragments were then washed in Dulbecco's PBS, Ca^{2+}- and Mg^{2+}-free medium (Sigma Chemical Co.), and mechanically dissociated. Single-cell suspensions were plated onto 24-well (2×10^5 cells/well) culture plates. The cells were incubated in F-10 medium supplemented with 10% horse serum, 4% FCS, and gentamycin (25 μg/mL) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. After 3 d, the medium was removed, and the cells were washed twice with serum-free F-10. The cells were then incubated in 1 mL of F-10 containing 0.1% BSA with no GH secretagogue or GHRP-6 (10^{-6}, 10^{-7}, 10^{-9} M) or GHRH (human GHRH-44, 10^{-8} M; Bachem, Philadelphia, PA) or GHRP-6 10^{-7} M plus GHRH 10^{-8} M. Media collected at the end of 15- and 30-min incubations were immediately frozen and stored at −20°C until assayed for GH content.

Pituitary GH Gene Expression

Total RNA was isolated by a single-step, acid guanidium thiocyanate-phenol-chloroform extraction method (19). Total RNA samples (20 μg/sample) were run on a 1.2% formaldehyde agarose gel and transferred to nylon membranes (Hybond N, Amersham, Little Chalfont, UK). The membranes were hybridized with a cDNA probe specific for rat GH (kindly provided by F. De Noto, University of California, San Francisco, CA). The cDNA probe was labeled by random primer (Megaprime, Amersham) with °α-^{32}PédCTP to a specific activity of 1×10^9 dpm/μg DNA. The nylon filters were rehybridized with a °α-^{32}PédCTP labeled rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe to control for homogeneity of RNA loading. Autoradiography was carried out at −70°C for 16–24 h with intensifying screens. Quan-

tification of hybridization signal was performed on a scanning densitometer (LKB XL, Laser Densitometer, Uppsala, Sweden). Pituitary GH mRNA levels were expressed as percentage of control group values.

GH Assay

GH concentrations in serum and in the pituitary culture media were measured by RIA with materials kindly provided by the National Institute of Diabetes, Digestive and Kidney Diseases of the National Institutes of Health. Values are expressed in terms of National Institute of Diabetes and Digestive and Kidney Diseases-rat-GH-RP-2 standard (potency 2 IU/mg) as μg/L of plasma or medium. The minimum detectable value of rat GH was 1.0 μg/L; intraassay variability was 6%. To avoid interassay variations, we assayed samples from each experiment within one RIA.

Statistical Analysis

Statistical differences were evaluated by the Dunnett's *t* test for multiple comparisons, preceded by analysis of variance. A p value less than 0.05 was considered significant.

RESULTS

Preliminary experiments had indicated that in rat pups serum GH levels peaked 20 min after the s.c. injection of GHRP-6 (data not shown). Therefore, blood samples from all subsequent studies were collected at that time. Administration of GHRP-6 elicited a marked ($p < 0.05$) rise in plasma GH concentrations at doses of 32, 150, 300, and 600 μg/kg (272, 327, 456, and 411% increase over saline-treated rats, respectively). The dose of 16 μg/kg did not stimulate GH secretion and that of 300 μg/kg resulted in a maximal response (Fig. 1).

GHRP-6 (10^{-6} and 10^{-7} M) stimulated GH release from primary pituitary cell cultures at 15 min (increase over basal levels, 47 and 37% for GHRP-6 10^{-6} and 10^{-7} M, respectively, $p < 0.05$); however, such stimulation was not observed at 30 min of incubation. At both time intervals, GHRP-6 (10^{-9} M) failed to stimulate GH release (Fig. 2). GHRH (10^{-8} M) alone or in combination with GHRP-6 (10^{-7} M) was effective in stimulating GH release at 15 (increase over basal levels, 43 and 52% for GHRH alone and GHRH + GHRP-6, respectively) and 30 min (47 and 52% for GHRH and GHRH + GHRP-6, respectively) (Fig. 2). The action of the two peptides was neither additive nor synergistic.

In vivo, 1 h after GHRH-ab administration, basal plasma GH levels were significantly reduced. No significant effect was evident on basal GH levels after treatment with somatostatin-ab or the combination of the two antisera. GH significantly increased in response to GHRP-6 (300 μg/kg, s.c.), and this response was not altered by antiserum pretreatment (Fig. 3).

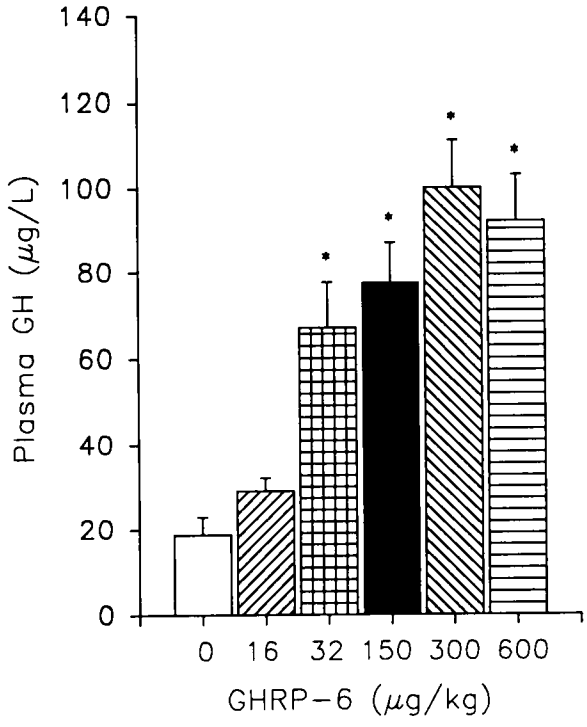

Figure 1. A representative dose-response curve of GHRP-6 on plasma GH concentrations in 14-d-old male and female rats. GHRP-6 (16, 32, 150, 300, and 600 μg/kg) or physiologic saline were administered s.c. 20 min before sample collection. Data are the mean ± SEM of nine replicates for each treatment group. Similar results were obtained in three similar independent experiments. *, $p < 0.05$ *vs* vehicle.

Passive immunization of GHRH from d 1 to d 10 of age significantly reduced pituitary GH mRNA expression (42% inhibition *versus* NRS + saline-treated pups; $p < 0.05$) (Fig. 4). GHRP-6 replacement therapy from d 8–10 of age resulted in only a slight increase in GH gene expression (21% increase *versus* GHRH-ab + saline-treated pups), but replacement therapy from d 6–10 resulted in full restoration of the GH mRNA expression to control levels (74% increase over GHRH-ab + saline-treated rats; $p < 0.05$). GHRP-6 treatment did not stimulate GH mRNA expression in the NRS-treated rats.

DISCUSSION

GHRP-6 elicited a marked, dose-related rise in plasma GH in infant rats. Its effects were apparently independent of endogenous GHRH and somatostatin release. Indeed, under our experimental conditions, functional blockade of the hypothalamic control of GH secretion by passive immunization with specific antisera against GHRH and somatostatin failed to alter GHRP-6-induced GH secretion. In infant rats after short-term GHRH deprivation, the hexapeptide was an effective stimulator of GH gene expression. However, in pituitary cultures GHRP-6 poorly and transiently stimulated GH release. Thus, we conclude that in infant rats: *1)* GHRP-6 is a potent stimulator of GH release; *2)* its action occurs primarily at the hypothalamic level; *3)* the mode of action of GHRP-6 is independent from the classic neuroendocrine control of

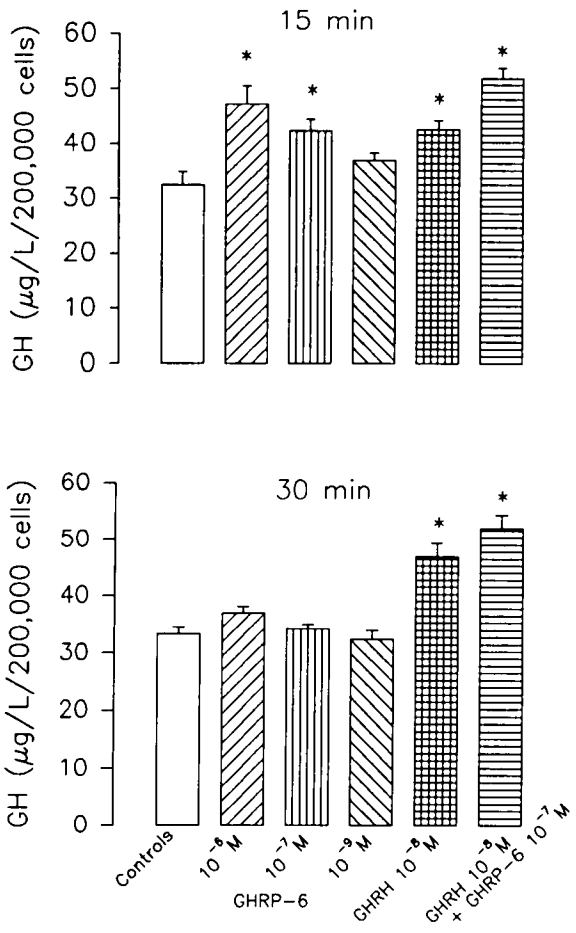

Figure 2. Effect of GHRP-6 and GHRH on GH release from 14-d-old rat pituitary cells. Cells (200,000 cells/well) were incubated with GHRP-6 (10^{-8}, 10^{-7}, and 10^{-6} M), GHRH (10^{-8} M), GHRP-6 (10^{-7} M) + GHRH (10^{-8} M) or medium alone for 15 or 30 min. Values are the mean ± SEM of six replicates and are representative of the results obtained in three similar independent experiments. *, $p < 0.05$ *vs* medium alone.

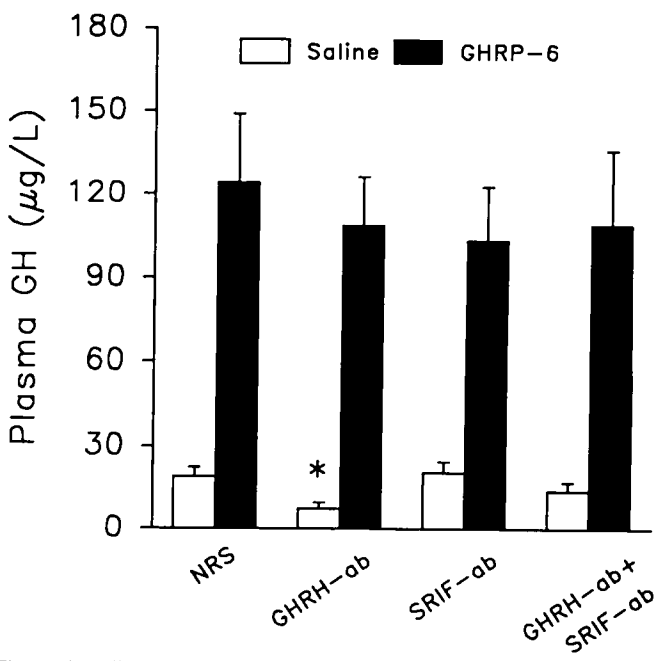

Figure 3. Effect of passive immunization with GHRH-ab and/or somatostatin-ab on GHRP-6 stimulated of GH secretion in 14-d-old rats. Groups of rats were injected with 100 μL NRS, 100 μL of GHRH-ab or somatostatin-ab, or 100 μL GHRH-ab + 100 μL somatostatin-ab 1 h before administration of GHRP-6 (300 μg/kg, s.c.) or physiologic saline. Blood samples were obtained at 20 min after GHRP-6 administration. Values are the mean ± SEM of six to eight replicates and are representative of the results obtained in three similar independent experiments. *, $p < 0.05$ *vs* NRS-treated rats.

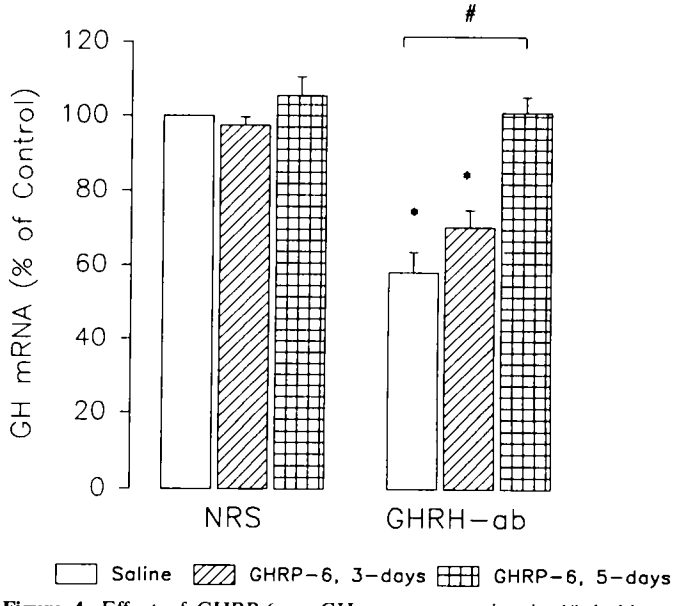

Figure 4. Effect of GHRP-6 on GH gene expression in 10-d-old rat pituitaries. Pups were given NRS or GHRH-ab (100 μL/rat/d) on d 1, 2, 4, 6, 8, and 10 of age. GHRP-6 (80 μg/kg, s.c., twice daily) or physiologic saline was administered from d 8 to 10 (3 d) or from d 6 to 10 (5 d). Data obtained from two independent similar experiments were evaluated by densitometry, expressed as a percentage of the NRS + saline group and pooled. n = 20–24 rats for each data point. *, $p < 0.05$ *vs* NRS + saline; #, $p < 0.05$ *vs* GHRH-ab + saline.

the hypothalamus; and *4)* GHRP-6 is a stimulator of GH gene expression in GHRH-deprived rats.

Our data in the infant rat contrast those obtained in adult rats. Clark *et al.* (11) and Bercu *et al.* (10) have shown that in the adult rat passive immunization against GHRH produced partial or nearly complete suppression of GHRP-6 activity, indicating that in the adult rat GHRH is a prerequisite for full expression of GHRP-6 activity. In this vein, it has been shown that systemic GHRP-6 administration activates subpopulations of GHRH neurons in the rat arcuate nucleus (20). However, several reports have been presented suggesting that GHRP-6 and GHRH operate by distinct receptors (4, 6, 21, 22).

Inhibition of endogenous somatostatin activity has also been proposed as a mechanism of GHRP-6 action in the adult rat (11) and in humans (23). However, in our hands depletion of hypothalamic somatostatin stores by cysteamine (24) did not impair the GH-releasing effect of GHRP-6 in adult rats (25). The reason for the discrepancies between these results and those of the present study may reside in the age of the animals studied. Infant rats, when compared with adult rats, are more sensitive to GH secretagogues (12, 13, 26–28) but less sensitive to the inhibitory control of somatostatin (29).

Proper interpretation of GHRP-6 mechanism of action is further compounded by recent data on GHRP-6 stimulation of GH secretion in rats with surgical ablation of the hypothalamus (25, 30) and in hypophysectomized rats bearing ectopic pituitary grafts (30). It is possible that GHRP-6 stimulates GH secretion *in vivo* through several pathways. For example, stimulation may involve some uncharacterized hypothalamic factor, some specific pituitary receptor, or some unknown peripheral factor. The relative weight of such mechanism(s) in mediating GHRP-6 action in infants rats may be different than in adult rats. It has been shown that stimuli that are ineffective on the adult rat pituitary, such as thyrotropin-releasing hormone, γ-aminobutyric acid, and galanin are potent GH-releasers in infant rats (26–28). Finally it must be considered that in our study the administration of GHRP-6 stimulated GHRH to such an extent that it overcame the neutralization capacity of the GHRH-ab. Such a hypothesis is unlikely because GHRP-6 was effective even when we doubled the GHRH-ab dose (data not presented).

To the best of our knowledge this is the first report on GHRP-6's ability to stimulate GH mRNA expression. That the hexapeptide is capable of stimulating GH mRNA expression in GHRH-ab treated rats is intriguing. A 3-d GHRP-6 treatment partially restored GH mRNA expression, and a 5-d treatment completely counteracted the effect of GHRH passive immunization. These data further substantiate the ability of GHRP-6 to stimulate somatotropic function independently from GHRH. However, GHRP-6 administration failed to stimulate GH gene expression in control pups. Two possible explanations for the latter results are possible. GHRP-6 may be able to stimulate GH mRNA transcription only when the GHRH-dependent GH gene expression is defective. Thus, GHRP-6 stimulation would not be revealed if GH gene expression is already maximally stimulated under the physiologic influence of GHRH. Alternatively, GHRP-6's ability to stimulate GH gene expression may have been masked in control rats by a concomitant reduction of the GHRH-induced transcription. It is conceivable that GHRP-6-stimulated GH secretion activates autofeedback mechanisms that reduce endogenous GHRH release (31). Regardless of the interpretation offered, the ability of GHRP-6 to take over the function of GHRH when the latter is defective stresses the potential of GHRP-6 or its analogues (32, 33) in the treatment of GHRH-deficient states.

It has been previously reported that GHRP-6 stimulates GH release from pituitary cells of adult rats in a dose-dependent manner, the maximum effect being achieved at a 10^{-7} M concentration (4). In our *in vitro* experiments, the stimulatory effect of GHRP-6 on GH release was prompt but of short duration. This indicates that in the neonatal rat the pituitary site of action only marginally contributes to the high GH response observed *in vivo*. It may be possible that at this age the GHRP-6 receptor, present in the adult pituitary (9), is not expressed or is functionally immature.

In conclusion, the present study indicates that in infant rats: *1)* GHRP-6 is a potent stimulator of GH release; *2)* it stimulates GH gene expression in the GHRH-deprived pup; *3)* its action is not mediated by GHRH or somatostatin; and *4)* its site of action is not on the somatotrophs.

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