Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 248-254

NEUROTENSIN REGULATION OF TSH SECRETION IN THE RAT

M.C. Sheppard, R.D. Askew, K.I.J. Shennan, S. Franks and D.B. Ramsden

Department of Medicine, University of Birmingham,
Birmingham, B15 2TH, U.K.

Received March 3, 1983

SUMMARY: Tri-iodothyronine stimulates neurotensin secretion from rat hypothalami *in vitro*. Propylthiouracil treatment of rats is associated with a decrease in anterior pituitary neurotensin content, an increase in hypothalamic content and an impaired release of peptide from hypothalami *in vitro*. In addition neurotensin has a direct inhibitory effect on TRH-stimulated TSH release from anterior pituitary *in vitro*. These observations suggest a physiological role for neurotensin in the control of TSH secretion in the rat.

Neurotensin is a tridecapeptide found in high concentration in hypothalamus, but with wide distribution throughout mammalian tissues, including anterior pituitary (1,2,3,4). Hormonal or neurotransmitter roles for neurotensin in central nervous system, anterior pituitary and gastro-intestinal tract have been proposed (5,6,7). The intraventricular injection of neurotensin in the rat reduces basal thyroid stimulating hormone (TSH) levels, the cold-induced TSH rise, the high circulating concentrations of TSH in thyroidectomised animals, and the TSH response to exogenous thyrotrophin releasing hormone (TRH) (7,8,9). This would suggest an inhibitory role for the peptide in regulating TSH secretion. If this regulatory effect is of physiological relevance, one might expect a feedback control mechanism involving thyroid hormones or TSH. The data that we present here indicate that tri-iodothyronine (T3) stimulates hypothalamic neurotensin release *in vitro*, that changes in hypothalamic and anterior pituitary neurotensin content occur in animals subjected to thyroxine (T4) or 6-propylthiouracil (PTU) administration, and that neurotensin has a direct inhibitory effect on TRH-stimulated TSH secretion *in vitro*.

0006-291X/83 $1.50

MATERIALS AND METHODS

Male albino Wistar rats (100-150 g at commencement) were used for the first set of experiments. Propylthiouracil-treated animals received 6-PTU (Sigma; 60 µg/ml) in drinking water for 9 weeks following a single subcutaneous injection of 25 µCi $Na^{131}I$. T4-treated rats received thyroxine (Sigma; 1 µg/ml) in drinking water for 10 weeks as well as alternate day subcutaneous injections of T4 (100 µg) for the first 4 weeks and daily injections during the 10th week. Control rats of similar weight were allowed normal drinking water ad libitum. At time of sacrifice animals were bled and serum T4 and TSH measured by radioimmunoassay (rat TSH reagents courtesy of National Pituitary Agency). Serum TSH concentrations were undetectable in T4-treated animals (< 0.08 µg/ml) and high in PTU-treated rats (4.90 ± 0.24 µg/ml) compared to controls (0.89 ± 0.12 µg/ml). Animals were killed by decapitation under light ether anaesthesia and hypothalamic blocks and pituitaries removed. Posterior pituitary was dissected free and tissues snap-frozen in liquid N_2 before storage at -70°C until extraction. Frozen tissues were thoroughly homogenized in 2.5 M acetic acid (1.5 ml per tissue sample) containing bacitracin (50 µg/ml), boiled for 15 min, rehomogenized, and the homogenate centrifuged at 1.7 x 10^3 g for 30 min at 4°C. Following centrifugation supernatant was decanted, frozen and lyophilized. Lyophilized extracts were resuspended and diluted in radioimmunoassay prior to measurement of neurotensin. Protein was measured in sodium deoxycholate (0.5%, w/v) solubilised tissue homogenate samples with the folin phenol reagent.

To measure release of neurotensin in vitro hypothalamic blocks were individually preincubated in 0.5 ml medium for 60 min. Medium comprised 116 mM NaCl, 5.36 mM KCl, 0.81 mM $MgSO_4$. $7H_2O$, 0.89 mM NaH_2PO_4. $2H_2O$, 1.79 mM $CaCl_2$, 10 mM glucose, 50 µg/ml bacitracin, 0.1% (w/v) bovine serum albumin and 20 mM Hepes (pH 8.1, Sigma). Following preincubation, medium was removed and replaced with either 0.5 ml normal medium or 0.5 ml test medium. Test substances used were T3 (Sigma; 10^{-9}, 10^{-8}, 10^{-7}M) reverse T3 (Sigma; 10^{-7}M), TRH (Universal Biologicals, Cambridge; 10 ng/ml, 100 ng/ml, 1 µg/ml), bovine TSH (Thytropar, Armour Pharmaceuticals; 10 µg/ml) and high K^+ medium (prepared by increasing KCl concentration to 58 mM and reducing NaCl to 58 mM). Incubation was continued for a further 30 min. All incubations were performed at 37°C in a shaking water bath in an atmosphere of 95% O_2 + 5% CO_2. Medium was frozen and stored at -20°C until assayed. Neurotensin was measured by radioimmunoassay as previously described (10) (antiserum kindly supplied by Dr. G. Bennett, University of Nottingham) with sensitivity 0.55 pg/tube. Logit-log plots of serial dilutions of immunoreactive neurotensin released from incubated hypothalamus and extracted from anterior pituitary and hypothalamus showed parallelism with decreasing amounts of synthetic neurotensin.

To determine the site of any inhibitory action of neurotensin on TSH secretion we have examined the direct effects of the peptide on the release of TSH from the anterior pituitary in vitro. Female albino Wistar rats (200-300 g) were killed by cervical fracture and the anterior pituitaries dissected, chopped into 0.5 mm^3 fragments and rinsed in ice-cold 25 mM Hepes buffer. Tissue fragments were mechanically agitated in Dulbecco's Modified Eagle's Medium (DMEM) containing collagenase (Worthington; 2 mg/ml) and Deoxyribonuclease (Sigma; 25 µg/ml) at 37°C for 30 min. The suspension of dispersed cells was centrifuged, resuspended and washed in DMEM. The resultant cell pellet was resuspended in DMEM supplemented with 10% foetal calf serum (FCS). Viability of cells was assessed by trypan blue exclusion and the cell suspension diluted with culture medium to approximately 4 x 10^5 viable cells/ml. Aliquots (250 µl) of this suspension were placed in a 96-well microtest plate (Nunclon) and incubated at 37°C in an atmosphere of 5% CO_2/95% O_2 (11). Incubation medium was replaced after 2 days with DMEM supplemented with 2.5% FCS. At 3 days incubation medium was replaced with either DMEM alone, DMEM + TRH (10 ng/ml), DMEM + Neurotensin (100 ng/ml) or DMEM + TRH (10 ng/ml) +

Neurotensin (100 ng/ml). After 2 hours incubation medium was removed and TSH concentration determined by radioimmunoassay. In a further set of experiments, pituitaries were removed from young adult euthyroid male rats, neural lobes were dissected away and the glands bisected. Hemipituitaries were chopped into 0.5 mm^3 blocks, mixed with Biogel P-2 in DMEM and loaded into disposable syringes (2 ml) adapted to form perifusion chambers. The tissue (2 hemipituitaries per column) was perifused with DMEM + 0.25% bovine serum albumin at 37°C at a rate of 0.5 ml/min. After 2 h equilibration pituitary fragments were exposed to TRH (10 ng/ml) followed by either a further control period of TRH stimulation (10 ng/ml) or by TRH plus neurotensin (50 ng/ml). TSH was measured in the effluent fractions (2 ml) and the TSH response to the first TRH stimulus compared with the response to the second stimulus for the same column.

RESULTS

The addition of 10^{-7} M T3 to incubation medium containing hypothalami removed from control rats significantly increased the release of neurotensin (45.3 ± 1.8 vs. 32.0 ± 1.9 pg/hypo/30 min, mean ± SEM, n = 10, $P < 0.005$). A dose-dependent response was observed from 10^{-7} M to 10^{-9} M T3 (50.1 ± 0.6, 46.7 ± 4.7 and 37.4 ± 2.9 vs 32.0 ± 2.9 pg/hypo/30 min in control incubations, n = 5). TRH, bovine TSH and reverse T3 had no effect on release of neurotensin. PTU administration markedly reduced anterior pituitary concentration of neurotensin (Table 1), confirming a previous report (4). Prolonged administration of T4 also significantly reduced anterior pituitary neurotensin (although to a lesser degree). Moreover, hypothalamic content was also significantly altered with high concentrations in PTU-treated

TABLE 1

Effect of altered thyroid status on neurotensin concentration of anterior pituitary and hypothalamus and on neurotensin release from incubated hypothalami.

	Anterior pituitary concentration (pg/mg protein)	Hypothalamic concentration (ng/mg protein)	Basal hypothalamic release (pg/hypo/30min)	KCl-stimulated hypothalamic release (pg/hypo/30min)
Control	613.0 ± 93.7 (10)	6.57 ± 0.37 (16)	18.1 ± 2.4 (8)	116.7 ± 5.4 (8)
PTU-treated	** 31.3 ± 3.7 (10)	**8.25 ± 0.27 (15)	16.6 ± 1.1 (8)	*90.2 ± 1.5 (7)
T4-treated	*290.2 ± 122.1 (9)	*5.77 ± 0.24 (18)	14.6 ± 0.5 (9)	106.3 ± 3.8 (9)

Results are expressed as mean ± standard error, number of experiments in parenthesis. Statistical differences were evaluated by Student's t test. * $P < 0.05$, ** $P < 0.001$ when compared to control.

animals and low concentrations in T4-treated rats (Table 1). Basal release over 30 min from incubated hypothalami was similar in the three groups of animals (Table 1), but the release from 'hypothyroid' hypothalami induced by 58 mM KCl (a potent depolarising stimulus) was reduced. Our studies with enzymatically dispersed anterior pituitary cells and perifused anterior pituitary fragments, have shown a direct inhibitory effect on TSH secretion in vitro. This response was confined to the TRH-induced release and no effect on basal secretion was observed (Table 2).

DISCUSSION

These data indicate a clear effect of thyroid hormones on hypothalamic neurotensin content and release. T3 stimulated neurotensin release

TABLE 2

a) Neurotensin effect on basal and TRH-stimulated TSH release from enzymatically dispersed euthyroid rat anterior pituitary cells. Results are expressed as mean ± standard error, number of experiments in parenthesis. Statistical differences were evaluated by Student's t test.

* P < 0.005 when compared to control

	TSH concentration in medium (ng/ml)
Control	114 ± 30 (8)
TRH (10 ng/ml)	*304 ± 66 (8)
Neurotensin (100 ng/ml)	95 ± 26 (8)
TRH (10 ng/ml)+ Neurotensin (100 ng/ml)	139 ± 37 (8)

b) Neurotensin effect on TRH-stimulated TSH release from perifused euthyroid rat anterior pituitary fragments. Responses to sequential stimuli of TRH are calculated from area under curve and expressed as mean ± standard deviation, number of experiments in parenthesis. Statistical differences were evaluated by two-tailed Wilcoxon sign rank test.

* P < 0.05 when compared to initial TSH response.

	TSH response (µg)	
	Control	Test
TRH (10 ng/ml)	8.4 ± 4.7 (6)	14.2 ± 11.6 (6)
TRH (10 ng/ml)	10.6 ± 7.5 (6)	-
TRH (10 ng/ml) + Neurotensin (50 ng/ml)	-	*8.1 ± 4.2 (6)

in vitro and long-term exposure of the hypothalamus to high levels of T3 in hyperthyroid animals caused depletion of neurotensin stores. The dependence of neurotensin release on thyroid hormones is illustrated by the increased hypothalamic concentration, and impaired potassium-induced release in vitro, in PTU-treated animals. Although indirect immunofluorescence has shown neurotensin to be present in some pituitary cells (4), its origin is uncertain and a hypothalamic source cannot be excluded. The pronounced reduction in anterior pituitary concentration of neurotensin in PTU-treated rats may therefore be consequent on reduced release of peptide into the hypophysial portal system in the absence of adequate circulating thyroid hormone concentrations. The hypercatabolic activity of hyperthyroidism may account for the lower levels in hypophysial tissue. Our data support the concept of a positive influence of T3 on hypothalamic release of neurotensin and have led us to the hypothesis that T3 exerts a negative feedback effect on its own secretion via neurotensin inhibition of pituitary TSH release.

To determine the site of this action we have examined the direct effects of neurotensin on the secretion of TSH by the anterior pituitary and our results show inhibition of TRH-stimulated TSH release. The lack of effect on basal hormone secretion contrasts with the observations of Vijayan and McCann (12), who reported enhanced release of TSH from incubated hemipituitaries in the presence of neurotensin at doses of 100 ng/ml or higher. Hypophysial portal blood secretion rates of TRH of 2.4 to 6.7 ng/hr have been reported recently (13), but neurotensin concentrations in portal blood are not known. Previous studies have shown that neurotensin stimulates somatostatin (a potent inhibitor of TSH secretion) release in vitro and in vivo (14, 15), and thus neurotensin may have a neuromodulatory role in the control of TSH both at hypothalamic and pituitary level. TRH antagonises a number of the central nervous system effects of neurotensin (8,9,16), and thus the interaction of TRH and neurotensin may not be limited to the anterior pituitary.

Thyroid hormones regulate TSH secretion by a direct effect on the pituitary, but in addition hypothalamic implants of T4 in the rat decrease thyroid hormone secretion and intrahypothalamic T3 injections reduce circulating TSH concentrations in hypothyroid monkeys (17,18,19). These data are compatible with a negative feedback effect of T3 on hypothalamic TRH release, but are also consistent with the hypothesis that the hypothalamic action of T3 is mediated by neurotensin. The in vitro effects of T3 on hypothalamic neurotensin release that we have reported are extremely rapid and may be related to specific T3 binding sites in the synaptosomal fraction of rat brain cells (20), suggesting that T3 has a neurotransmitter or neuromodulator role at nerve terminals. The negative feedback action of T3 could therefore be mediated by hypothalamic TRH, hypothalamic neurotensin acting at the pituitary, or hypothalamic neurotensin acting via somatostatin release. All three modes may exist in parallel forming an integrated and powerful system to ensure precise regulation of thyroid hormone secretion.

ACKNOWLEDGEMENTS

Financial assistance for these studies was provided by the West Midlands Regional Health Authority, Medical Research Council (G979/1173/SB) and the Central Birmingham Health District Endowment Fund. M.C.S. is in receipt of a Wellcome Trust Senior Lectureship.

REFERENCES

1. Carraway, R. and Leeman, S.E. (1973) J. Biol. Chem. 248, 6854-6861.
2. Cooper, P.E., Fernstrom, M.H., Rorstad, O.P., Leeman, S.E. and Martin, J.B. (1981) Brain Res. 218, 219-232.
3. Polak, J.M., Sullivan, S.N., Bloom, S.R., Buchan, A.M.J. , Facer, P., Brown, M.R. and Pearse, A.G.E. (1977) Nature 270, 183-184.
4. Goedert, M., Lightman, S.L., Nagy, J.I., Marley, P.D. and Emson,P.C. (1982) Nature 298, 163-165.
5. Uhl, G.R. and Snyder, S.H. (1976) Life Sci. 19, 1827-1832.
6. Blackburn, A.M. and Bloom, S.R. (1979) J. Endocrinol. 83, 175-181.
7. Maeda, K. and Frohman, L.A. (1978) Endocrinology 103, 1903-1909.
8. Nemeroff, C.B., Bissette, G., Manberg, P.J., Osbahr, A.J., Breese, G.R. and Prange, A.J. (1980) Brain Res. 195, 69-84.
9. Nemeroff, C.B., Luttinger, D. and Prange, A.J. (1980) Trends Neurosci. 3, 212-215.
10. Shennan, K.I.J. and Sheppard, M.C. (1983) J. Endocrinol. (in press)
11. Daniel, S. and Ramsden, D.B. (1982) J. Endocrinol. 95, 229-235
12. Yijayan, E. and McCann, S.M. (1980) Life Sci. 26, 321-327.

13. Fink, G., Koch, Y. and BenAroya, N. (1982) Brain Res. 243, 186-189.
14. Sheppard, M.C., Kronheim, S. and Pimstone, B.L. (1979) J. Neurochem. 32, 647-649.
15. Abe, H., Chihara, K., Chiba, T., Matsukura, S. and Fujita, T. (1981) Endocrinology 108, 1939-1943.
16. Osbahr, A.J., Nemeroff, C.B., Manberg, P.J. and Prange, A.J. (1979) Eur. J. Pharmacol. 54, 299-302.
17. Kajihara, A. and Kendall, J,W. (1969) Neuroendocrinology 5, 53-63.
18. Chambers, W.F. and Sobel, R.J. (1971) Neuroendocrinology 7, 37-45.
19. Belchetz, P.E., Gredley, G., Bird, D. and Himsworth, R.L. (1978) J. Endocrinol. 76, 439-448.
20. Mashio, Y., Inada, M., Tanaka, K., Ishii, H., Naito, K., Nishikawa, M., Takahashi, K. and Imura, H. (1982) Endocrinology 110, 1257-1261.