Neurochemical Research, Vol. 19, No. 7, 1994, pp. 845–850

In Vitro TRH Release from Hypothalamus Slices Varies During the Diurnal Cycle

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(Accepted February 28, 1994)

We have previously described a daily rhythm in thyrotropin releasing hormone (TRH) and TRH mRNA in the rat hypothalamus. To determine whether TRH release fluctuates in a diurnal manner, we have measured basal and potassium stimulated release from hypothalamic slices, and compared it to release from olfactory bulb slices, during the diurnal cycle. Basal TRH release was higher at 7:00 h than at any other time (1:00, 13:00 or 19:00 h) in either hypothalamus or olfactory bulb. The ratio of stimulated over basal release was higher in the hypothalamus at 19:00 h, when TRH content was highest. Potassium stimulated TRH release from olfactory bulb was not different from basal release at any time. TRH release fluctuations were not due to a rhythm of extracellular inactivation: the activity of pyroglutamyl aminopeptidase II, an ectoenzyme responsible for TRH inactivation, was constant throughout the cycle. Our data demonstrate that diurnal variations of TRH release occur in vitro and that the enhanced responsiveness to potassium stimulation in hypothalamus is correlated with increased levels of peptide.

KEY WORDS: Thyrotropin releasing hormone; hypothalamus; olfactory bulb; in vitro release; circadian cycle.

INTRODUCTION

Neuropeptide expression involves several steps including biosynthesis, processing, and compartmentalization, that eventually set the level of active peptide released in response to specific stimuli. Actual peptide release in vivo, a consequence of peptidergic activity, has been difficult to estimate. It is possible to access to the extracellular medium by using in vivo techniques (1,2), but these methods are technically complicated. In vitro systems have provided useful tools to test, not only the type of receptors or second messengers involved in release regulation (3,4) but, in a few cases, as a mean to approximate in vivo release or to study cell responsiveness. For example, luteinizing hormone-releasing hormone release induced by systemic injection of progesterone in ovariectomized, oestradiol-primed female rats can be detected either in vivo (5) or in vitro (6). Measure of peptide release in conditions (i.e., high KCl concentration) that cause massive exocytosis, can be inferred as a quantification of releasable pools and/or cellular responsiveness.

Thyrotropin releasing hormone (TRH) controls the release of two pituitary hormones, thyrotropin (TSH) and prolactin (PRL), and probably has neuromodulatory functions in central nervous system (CNS) areas (7). It is released form median eminence in response to hypothyroidism (8), cold stress (9) or suckling (10). In these conditions, TRH release in vivo is associated with increased TRH mRNA levels in the paraventricular nucleus (PVN) (11,12). We have previously shown that, during the diurnal cycle, the hypothalamic levels of TRH

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0364-3190/94/0700-0845$07.00/0

vary in coincidence with those of its mRNA as well as the reported serum TSH levels (13). This suggests that hypothalamic TRH release in vivo fluctuates during the diurnal cycle. This has also been observed for other neuropeptides such as propiomelanocortin (14), corticotropin releasing factor (15) and vasopressin (16).

To obtain a better understanding of the functional relationship between synthesis and release in TRHergic neurons, we measured in vitro TRH release from hypothalamus in basal and depolarizing conditions during the diurnal cycle. Data were compared with release from olfactory bulb since a previous study demonstrated a lack of response to depolarization between 10:00 and 12:00 h in that region (17). Our results reveal that high hypothalamic TRH levels in vivo correlate with high TRH release responsiveness to depolarization in vitro but not with basal release that is higher when content is lower. We also show that in olfactory bulb the basal release rhythm is similar to that in hypothalamus. However, olfactory bulb did not respond to depolarization at any time. Moreover, we demonstrate that these rhythms are not due to a daily variation of the activity of pyroglutamyl aminopeptidase II (PPII) which is the enzyme involved in TRH extracellular inactivation in brain (18,19).

EXPERIMENTAL PROCEDURE

Animals. We used male Wistar rats (60 days old) maintained in a 12 h light-dark period (lights on at 6:00 h); rat chow and water were provided ad libitum. Each group of rats was sacrificed by decapitation within less than 30 min in the room next to the one they were kept in. During dark phase, animals were sacrificed under dim red illumination. The same pool of animals was used to measure tissue and released TRH.

Release Experiments. We performed in vitro release experiments as described by Méndez et al. (17). Briefly, hypothalamus and olfactory bulb were dissected and sliced (250 μm) in a tissue chopper (Brinkman). Sliced tissues were transferred into a cut 20 ml plastic syringe with a nylon mesh (63 μm) at the bottom. Each sample was washed 3 times in Krebs-Ringer bicarbonate solution (KRB: 125 mM NaCl, 4.4 mM KCl, 1.2 mM KH_2PO_4 1.3 mM $MgSO_4$, 2.6 mM $NaHCO_3$, 10 mM glucose, 2.5 mM $CaCl_2$ (pH 7.4) and gazed with 95% O_2-5% CO_2) at room temperature. Tissues were then incubated in a 20 ml vial containing 1.5 ml KRB for 10 min at 37°C and this procedure repeated 5 times; TRH release to the 6th vial (i.e., 50-60 min incubation) was considered the basal release. Finally, tissues were incubated for 10 min in a vial containing KRB with 56 mM KCl; TRH released under these conditions was considered the stimulated release. Immediately after each incubation, methanol (75% final) was added to the medium and kept at −20°C. Tissues were immediately frozen on dry ice and stored at −70°C.

TRH Extraction and Radioimmunoassay. TRH extraction and radioimmunoassay were done as described previously (17).

PPII Activity. PPII specific activity was measured as reported (20).

Statistical Analysis. Student's *t*-test was used to compare differences between groups. Differences were considered significant at $p < 0.05$.

RESULTS

We have previously shown that TRH content in the hypothalamus varies during the diurnal cycle, with a peak at 19:00 h (13). We confirmed those data and showed no significant day-time variations in olfatory bulb TRH content (Fig. 1) Hypothalamic or olfactory bulb slices, from animals sacrificed at different times (as in Fig. 1), were used to measure TRH release in basal and 56 mM K^+ stimulated conditions. In hypothalamus, release decreased from high levels at beginning of incubation to

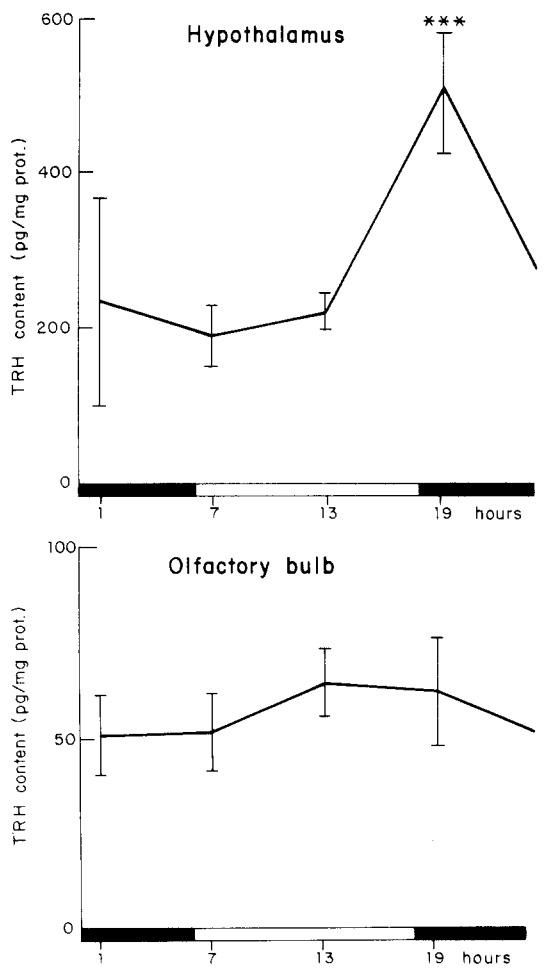

Fig. 1. TRH diurnal variations in hypothalamus and olfactory bulb. TRH was quantified by radioimmunoassay as reported earlier (17). Bars represent the standard error of the mean (n = 6). ***: $p < 0.01$ compared to 13:00 h.

stable levels before stimulation with KCl (Fig. 2). In olfactory bulb, the decline was less evident (Fig. 3). In both regions, basal TRH release varied according to the hour of sacrifice (Fig. 2 and 3): a peak was observed at 7:00 h in hypothalamus and olfactory bulb (Fig. 4A and 4B). In response to potassium depolarization, a statistically significant increase of TRH release was detected in hypothalamus at 13:00 and 19:00 h (Fig. 2) whereas olfactory bulb did not respond at any time (Fig. 3). In hypothalamus, the ratio of stimulated/basal release varied depending on the time of day; maximal response was observed at 19:00 h (Fig. 4A). These data were essentially similar to those of other experiments in which we studied TRH release at 1:00, 7:00, 13:00 and 19:00 h (experiment 2) or at 7:00 and 19:00 h (experiment 3) (not shown).

To exclude the possibility that the variations observed are due to extracellular TRH degradation, we measured the activity of PPII, the membrane-bound ectopeptidase with narrow specificity for TRH (18). Tissues were sampled at 2:00, 8:00, 14:00 and 20:00 h. PPII activity did not change during the diurnal cycle in hypothalamus (Table I) or olfactory bulb (not shown).

DISCUSSION

We have previously observed differences in the ratio of basal TRH release in vitro over content or in the ratio of potassium stimulated over basal TRH release among brain regions (17). These results suggest that various intracellular TRH pools exist within specific neurons. Here we report that differences are not only spatial-specific but also temporal-specific. Hypothalamus showed a clear diurnal rhythm in stimulated TRH release that was similar to that previously observed with the diurnal

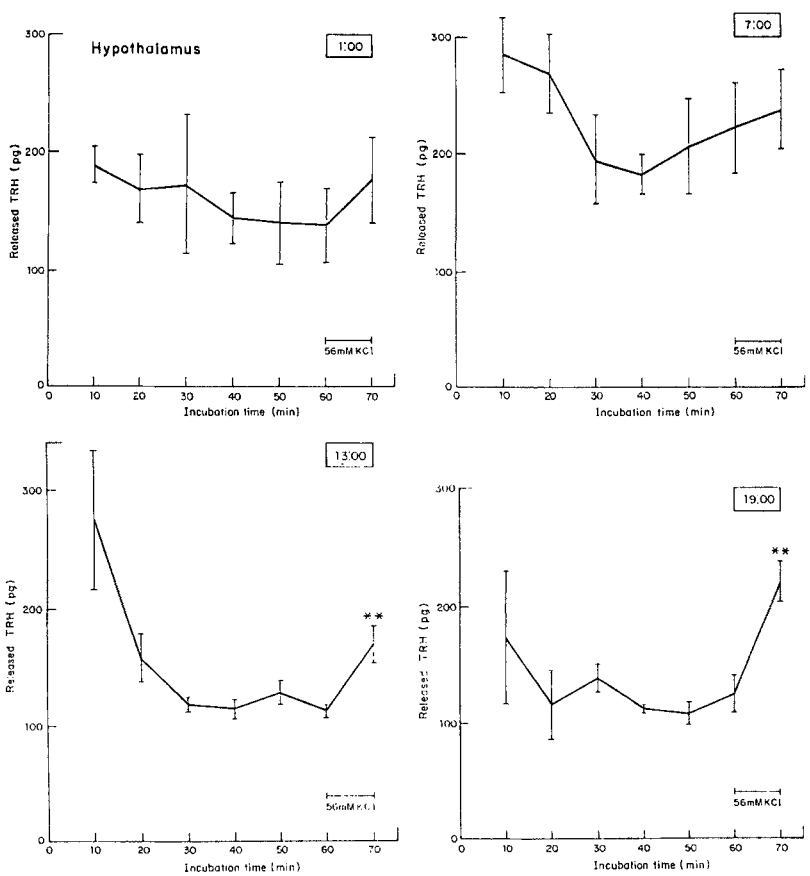

Fig. 2. In vitro TRH release by hypothalamus during the diurnal cycle. Tissue samples from the same animal pools as for Fig. 1 were sliced and incubated in KRB medium for 60 min. KRB was replaced with fresh medium every 10 min (last 10 min incubation corresponds to basal release). Tissues were ultimately incubated 10 more min in 56 mM KCl KRB buffer (stimulated release). Bars represent the standard error of the mean (n = 6). **: $p < 0.02$ compared to basal release.

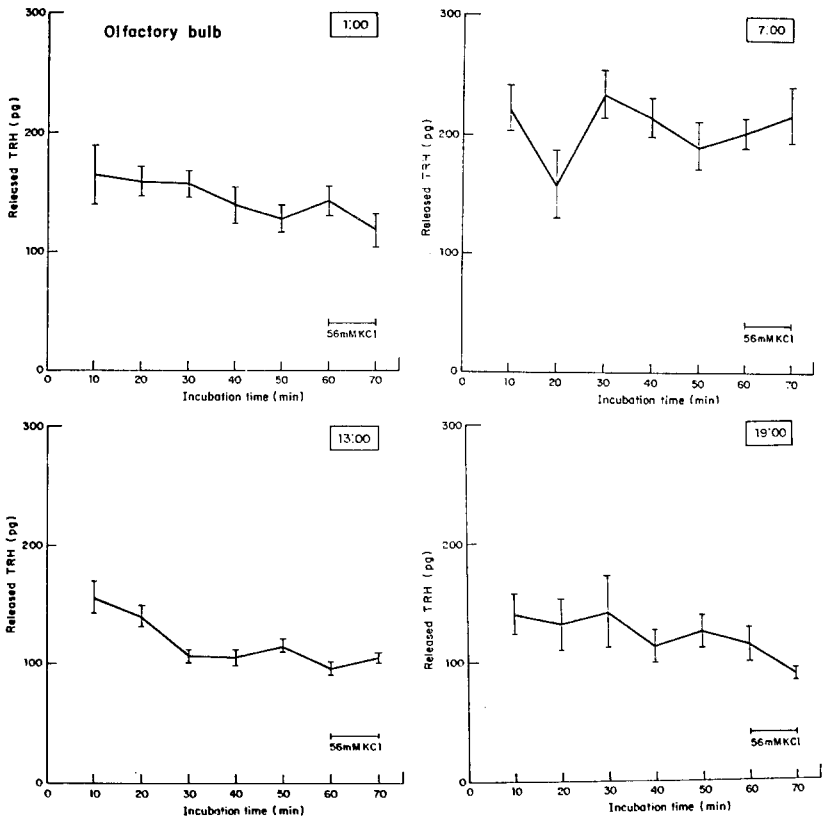

Fig. 3. In vitro TRH release by olfactory bulb during the diurnal cycle. Tissue samples from the same animal groups as for Fig. 1 were sliced and incubated as described in Fig. 2. Bars represent the standard error of the mean (n = 6).

pattern of TRH mRNA and peptide levels (13). Additionally, hypothalamus and olfactory bulb displayed a diurnal pattern of basal release opposite to the hypothalamic stimulated cycle.

The exact nature of events that establish the levels of TRH detected in the extracellular fluid in vitro is not yet known. Basal release may include artifactual leakage as well as the releasing activity of neurons. The existence of release fluctuations suggests that a physiological mechanism determines their amplitude. Further evidence that TRH leaking from tissue is not significantly contributing to basal release is that it was higher when tissue content was lower in hypothalamus. Stimulated release may give an index of the size of the releasable pool and/or the state of responsiveness of the neuron to stimuli.

The levels measured in extracellular fluid are the resultant of the balance between release and inactivation. We can exclude that the variations we detected are due to changes in TRH extracellular degradation, since we did not observe a significant diurnal cycle of pyroglutamate aminopeptidase II, the enzyme competent for inactivation of released TRH (18,19).

We may interpret our data in two ways. First, TRH basal release may be an index of TRH in vivo release, as implied from studies comparing in vivo and in vitro release. Therefore, our studies may suggest that in vivo TRH release is high at 7:00 h and low at 19:00 h. In hypothalamus the high release rate may have depleted the releasable pool of TRH at 7:00 h so that the potassium stimulus was not able to increase TRH release; this situation would be reversed at 19:00 h. In the olfactory bulb, cells were not responsive to high potassium at any time, presumably because values of basal release over tissue content are much higher than in hypothalamus, even when basal release is low, implying that the releasable pool was constantly low (see also reference 17).

Alternatively, fluctuations in basal release may not be responsible for depleting the releasable pool so that response to depolarization would not relate preferentially to the size of the pool but to the state of responsiveness to the stimulus. In this case, two models may explain the fluctuating response to depolarization in the hypothalamus. One would require a synergistic factor necessary for the response to depolarization. A second

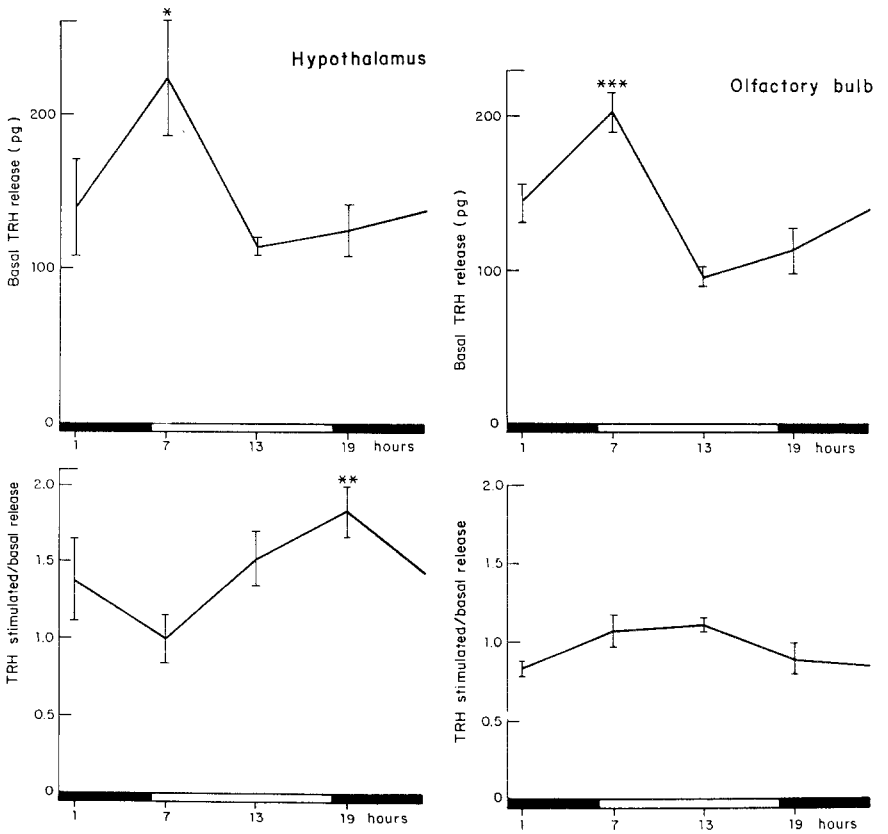

Fig. 4. Diurnal variations of basal and potassium-stimulated/basal in vitro TRH release. Basal release represents the amount of TRH released in the last 10 min incubate (i.e., between 50 and 60 min in Fig. 2 and 3). Potassium-stimulated/basal release is the ratio between amount of TRH released in the high potassium incubate and amount released in the previous incubate for each sample. Bars represent the standard error or the mean (n = 6). *: $p < 0.05$; ***: $p < 0.01$ compared to 13:00 h.

Table I. Hypothalamic PP II Specific Activity During the Diurnal Cycle

	PP II specific activity	
Time of day	1st experiment	2nd experiment
2:00	1.78 ± 0.09	1.21 ± 0.09
8:00	1.59 ± 0.02	1.18 ± 0.10
14:00	1.49 ± 0.09	1.33 ± 0.09
20:00	1.58 ± 0.18	1.21 ± 0.08

Hypothalami were individually homogenized and membranes isolated and preincubated in 50 mM $NaHPO_4$ pH 7.5 with bacitracin and N-ethylmaleimide (2.5 mM final) at 37°C for 5 min. At t=0 200,000 cpm [^{3}Hpro]-TRH (10^{-6} M) were added and aliquots taken at various times. His-[^{3}Hpro]NH_2 produced was separated from [^{3}Hpro]-TRH by ion exchange paper chromatography. Activity was linear with time and protein concentration. Data are mean ± SEM (n=5) pmoles his-pro-NH_2/min/mg protein.

possibility would involve diurnal variations of an inhibitory factor that impedes response to depolarization. Inhibitory neurotransmitters to TRH release in hypothalamus have been identified (21,22) consistent with the latter model. In addition, data on diurnal control of potassium-stimulated release of vasoactive intestinal peptide showed an adrenergic-mediated inhibitory mechanism (23). It is then possible that the endogenous-clock controlled mechanism is an inhibitory factor that, when on (primary stimulus), changes responsiveness to different secondary stimuli. In this model, most TRH-ergic hypothalamic nuclei would be synchronized by the inhibitory stimulus. Since slices were used in the present experiments, regulation of potassium-induced TRH release during diurnal cycle in hypothalamus is better explained by regulation of intracellular mechanisms in response to different stimuli. An endogenous clock that drives periodicity may regulate at least one of these stimuli. Regulation of basal release in both hypothalamus and olfatory bulb could be due to a different but interacting mechanism since an inverse pattern was observed in hypothalamus between basal and stimulated release. According to this second

model, the diurnal regulatory mechanism (conceivably an inhibitory neurotransmitter) may cause the changes in basal release.

If the second interpretation is correct, our data imply that in hypothalamus the sensitivity to stimuli is correlated with increased biosynthesis. In conclusion, although it will be necessary to perform in vivo measurement of release to confirm this hypothesis, our in vitro release data suggest that in hypothalamus a diurnal rhythm of TRH release exists and that coordinated stimuli control TRH expression and release.

ACKNOWLEDGMENTS

The authors thank M. Cisneros for technical assistance and S. González and E. Mata for animal care. This research was partially supported by grants from CONACYT (ICSAXNA 030915) and EEC (CL1356F).

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