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NEURONAL TRH SYNTHESIS: DEVELOPMENTAL AND CIRCADIAN TRH mRNA LEVELS

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SUMMARY: Peptide biosynthesis within a neuron involves several steps occurring at the soma and during its travel to the nerve terminal, where it accumulates to be released under stimulatory conditions. We have measured hypothalamic TRH and TRH mRNA during ontogeny and circadian cycle and observed that TRH mRNA variations are more prominent than TRH ones. On the basis of these results and in vitro release experiments, we propose a compensatory mechanism working at the nerve terminal which is activated after release.

When a peptide is released in response to endogeneous or environmental stimuli, a mechanism for recovering the basal intracellular levels must exist. Although many complex regulatory processes might be involved, biosynthesis and/or intracellular degradation rate can play an important role. Several steps are involved in peptide biosynthesis where mRNA levels can be a limiting component. Peptide biosynthesis has been shown to be regulated at transcriptional level by the same effectors that stimulate release (1,2,3); for POMC, other steps such as postranslational processing and peptide modifications are also affected (4,5).

TRH is a tripeptide with both endocrine and neural functions (for review see 6). The sequence for TRH precursor has been deduced from a cDNA clone (7) and TRH mode of degradation has also been studied (8,9,10). In this report, we focus on TRH and its specific mRNA levels in the hypothalamus in two different conditions, ontogeny (11) and circadian cycle (12), as an approach to study in vivo overall kinetics of TRH metabolism. Our data show evidence for a coupling between biosynthesis and release and gives insights for an intracellular compensatory mechanism.

MATERIALS AND METHODS: Male Wistar rats, fed ad libitum (Purina Chow) were maintained in a 12 h light-dark cycle (light on 6:00-18:00). All rats at each time were sacrificed within an hour and tissues were dissected and kept frozen (-70 °C) until assayed.

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Each hypothalamus was homogeneized in 200 ul 50mM Tris-HCl pH 7.4, 25 mM NaCl, 5mM MgCl; 8ul of 25% Triton X-100 and 100 ul of 72% sacarose were added and the homogenate centrifuged 10 min at 4°C; the supernatant (cytosolic fraction) where 100 ul of 6% SDS, 0.4M NaCl, 40mM EDTA solution were added, was extracted 3x with phenol-chloroform (vol./vol); to the aqueous phase, 5M NaCl (1/25th of total vol.) and 2 vol. of ethanol were added and left at -70°C for 12 h to precipitate total RNA. RNA samples were verified on 1% agarose minigels (containing 2.2M formaldehyde, 10mM sodium phosphate buffer, pH 7).

TRH cDNA kindly donated by Dr. R. Goodman (7) was [^{32}P]-labeled by Nick Translation to 1-5x10^8 cpm/ug specific activity. RNA samples were run in formaldehyde minigels and transfered to nitrocellulose paper. Hybridization was performed at 42°C in 5x SSC, 1x Denhart, 20mM sodium phosphate pH 6.5, 10% dextran sulphate and 50% formamide (13). Ribosomal RNA bands (stained with ethidium bromide) and TRH-mRNA autoradiographic band were quantified by densitometry in a Hoeffer scanning densitometer GS300. All data were measured within the linear range of detection.

TRH was quantified by specific radioimmunoassay according to Mendez et al (14).

RESULTS AND DISCUSSION

To determine whether TRH mRNA changes concomitantly with TRH levels within the hypothalamus, we measured both parameters during ontogeny (Fig. 1). TRH mRNA levels showed same increasing tendency as TRH within the first 20 days postnatal but, later in development, some differences were seen between TRH and TRH mRNA behaviour. Therefore, at least in part, TRH mRNA is responsible for TRH levels during ontogeny.

Several authors have reported the ontogenetic pattern of TRH content (11,15,16). Looking at curve pattern all reports are similar up to 20-30 days of age and differences are seen later in development; our results resemble those of Martino et al (11) and Gayo et al (15). These last authors have made an analogy in ontogenesis of TRH, TSH and thyroid hormones. Within the hypothalamus-hypophysis-thyroid axis variations in TRH mRNA levels can be due essentially to two processes: cellular development of hypothalamus, specifically TRH-ergic neurons, and negative feedback by thyroid hormones on TRH biosynthesis (17,18). The TRH mRNA fall on day 45 might be due to the accumulation of thyroid hormones at this period.

Although TRH role on TSH secretion is well documented, it is not clear how much secreted TRH is involved in other endocrine functions (e.g. prolactin secretion). Hypothalamic TRH can have other non-endocrine functions (e.g. neurotransmitter) from those TRH-ergic neurons which do not direct axons to the median eminence. Therefore, the pattern for TRH and TRH mRNA observed can be due to different physiological phenomena occurring in the developing rat (e.g. circadian rhythm maturation, puberty) as well as different regulatory mechanisms working in each group of neurons involved. Microdissection of hypothalamic nuclei will help to get a more homogeneous population of TRH-ergic neurons.

TRH release is a rapid event causing a depletion of intracellular TRH and

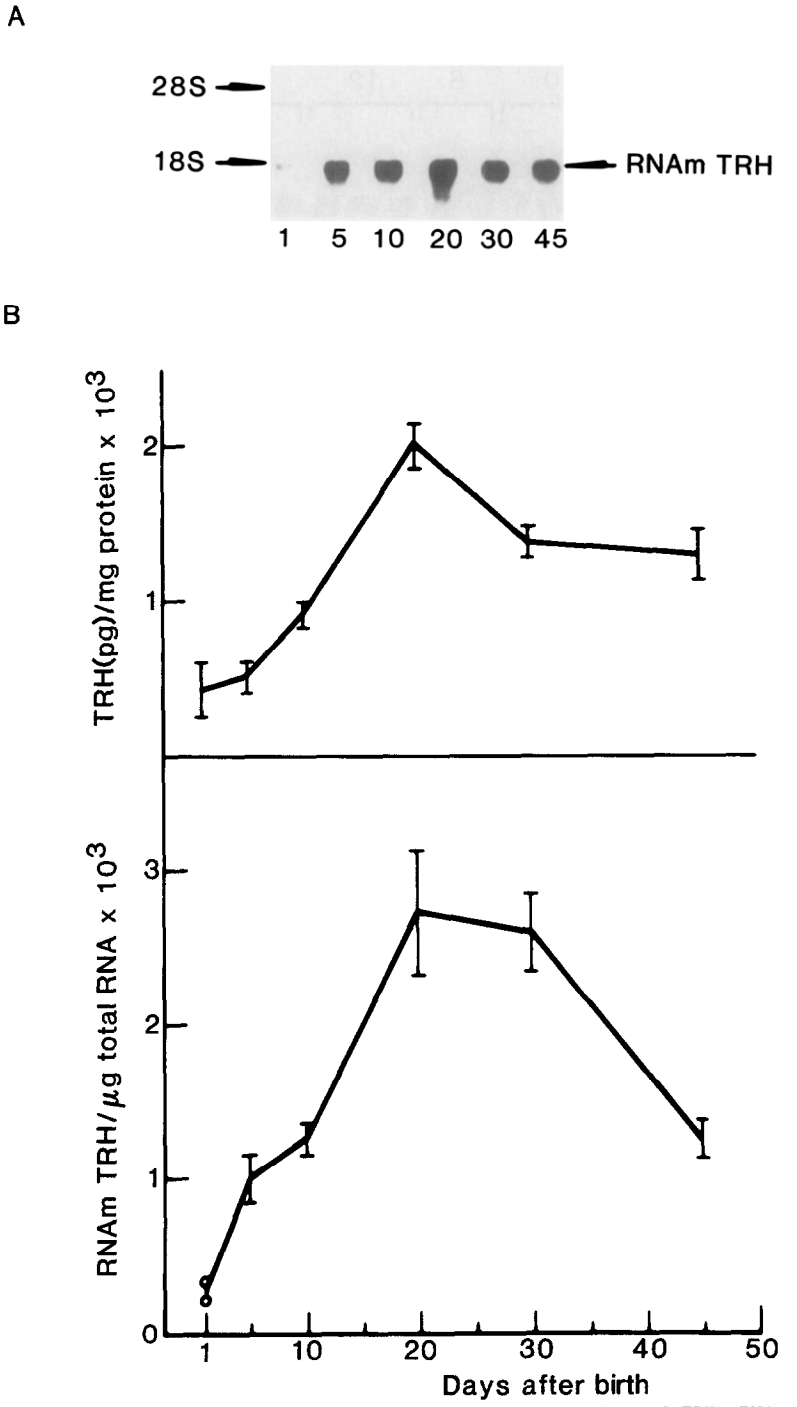

Fig. 1. TRH and TRH mRNA during ontogeny. Hypothalamic TRH and TRH mRNA were measured at different days after birth as indicated in Materials and Methods. (A) Autoradiography of representative RNA samples hybridized with TRH cDNA [^{32}P]-labeled; note that expected size (1.6 Kb) is seen at all ages. (B) TRH and TRH mRNA were normalized per mg of protein and ug of total RNA respectively. Each value is the mean ± SEM (n=3).

the need for a mechanism responsible for recovering basal levels. In order to study the role of mRNA on adjustment of hypothalamic TRH levels, we measured TRH mRNA together with TRH during circadian cycle (Fig. 2). The observed

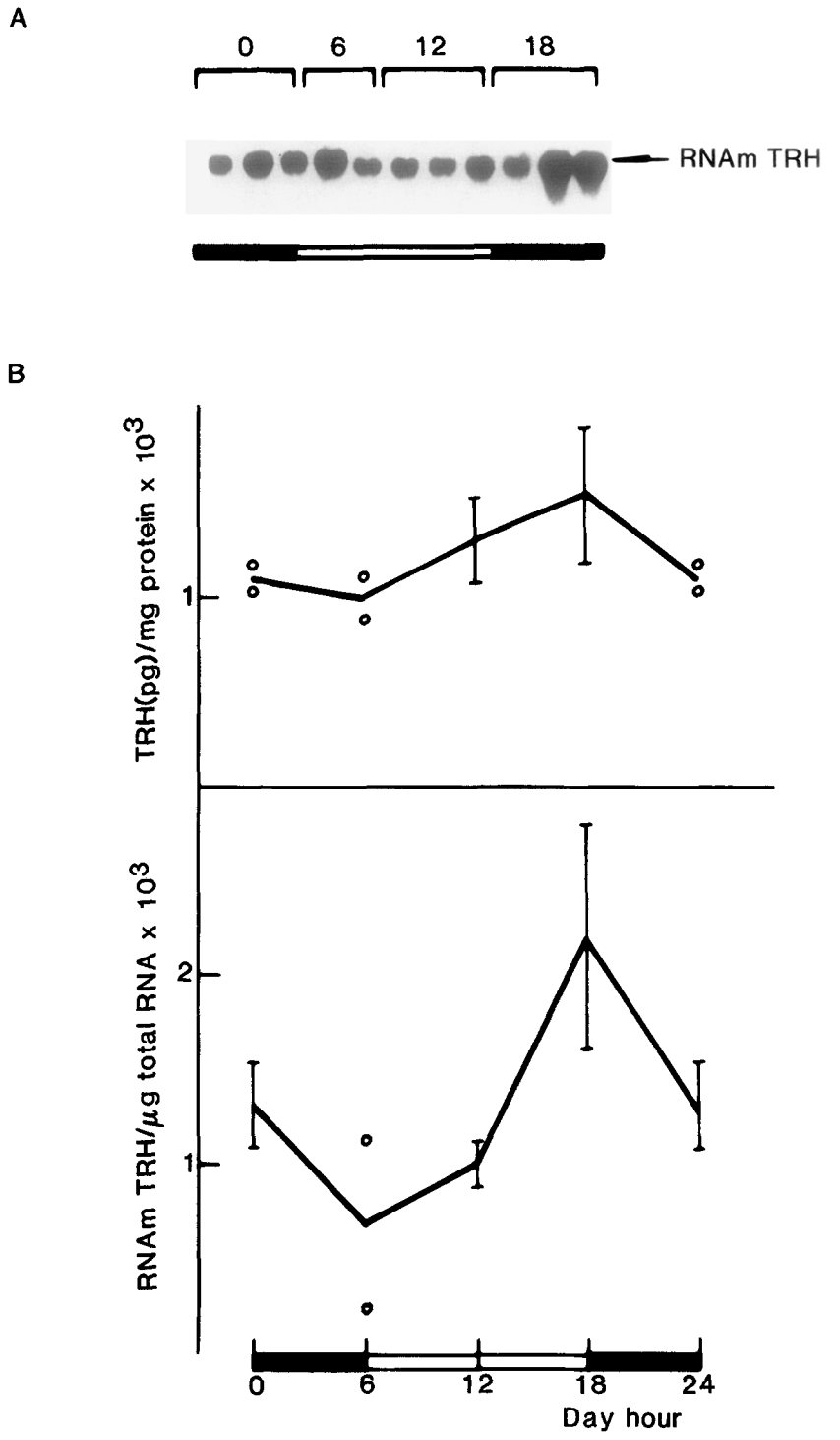

Fig. 2. TRH and TRH mRNA during circadian cycle. Hypothalamic TRH and TRH mRNA of adult rats were measured at different time of the day as indicated in Materials and Methods. (A) Autoradiography of RNA samples hybridized with TRH cDNA [^{32}P] labeled. (B) TRH and TRH mRNA were normalized per mg of protein and ug of total RNA respectively. Mean ± SEM (n=3).

circadian rhythm of TRH and its mRNA were similar in adult animals, with highest levels at 18 hrs. These results suggest that TRH release during light-dark cycle is closely coupled with TRH mRNA levels within the 24 hrs. period.

TRH circadian pattern observed is similar to the ones described before (12,19). TRH peak appears just before the activity period of rats and falls near its end. This result correlates well with TSH circadian rhythm, but as mentioned before, hypothalamic TRH is involved in other no well defined functions, some of which might have circadian cycles too. The well defined rhythm in the whole hypothalamus suggests that most of TRH-ergic cycling nuclei are syncronized; this hypothesis is supported by Kerdelhue's results (19) showing two hypothalamic areas with maximum values at around the same time.

Since we found fluctuations in TRH as well as TRH mRNA levels during circadian cycle, and Martino et al (12) have shown a developmental rise of TRH rhythm, we decided to analyze how much dependency exist between circadian rhythm and postnatal development for defining peptide and mRNA levels. Fig. 3 shows a variety of circadian patterns for TRH and TRH mRNA at different ages ranging from no fluctuations (TRH at 5 days) to more than one peak (TRH mRNA at 30 days). These data indicate that TRH and TRH mRNA levels depend on both postnatal development and circadian rhythm behaviour of TRH-ergic neurons; therefore, they are a combination of biological and physiological processes.

The development of TRH circadian rhythm which is attained only at adult stage, suggest that it is a consequence of multifactorial influences that only when all are achieved, it becomes sinchronized. It is difficult, and would be to speculative to stress which of different (if no all) factors can affect (such as neuronal, hormonal- i.e. puberty-or behavioural).

TRH biosynthesis involves all steps of protein synthesis plus postranslational processing and terminal modifications (pGlu formation and amidation). As shown, during ontogeny, between days 1 and 20 TRH mRNA increased 10 fold and TRH only 5 fold and, during circadian cycle, TRH mRNA increased 3x compared to the bare increase of TRH. These observations imply that although correlations exists between intracellular TRH and TRH mRNA in the phenomena described, compensatory mechanisms might also be involved in setting the final TRH levels.

Several steps could be regulated after transcription: mRNA stability, translation and postranslational events. We have not established if changes in mRNA levels observed are due to the transcription process itself or mRNA stability; direct measurement of transcription rate will define which process dominate mRNA levels in our conditions. On the other hand, experiments should

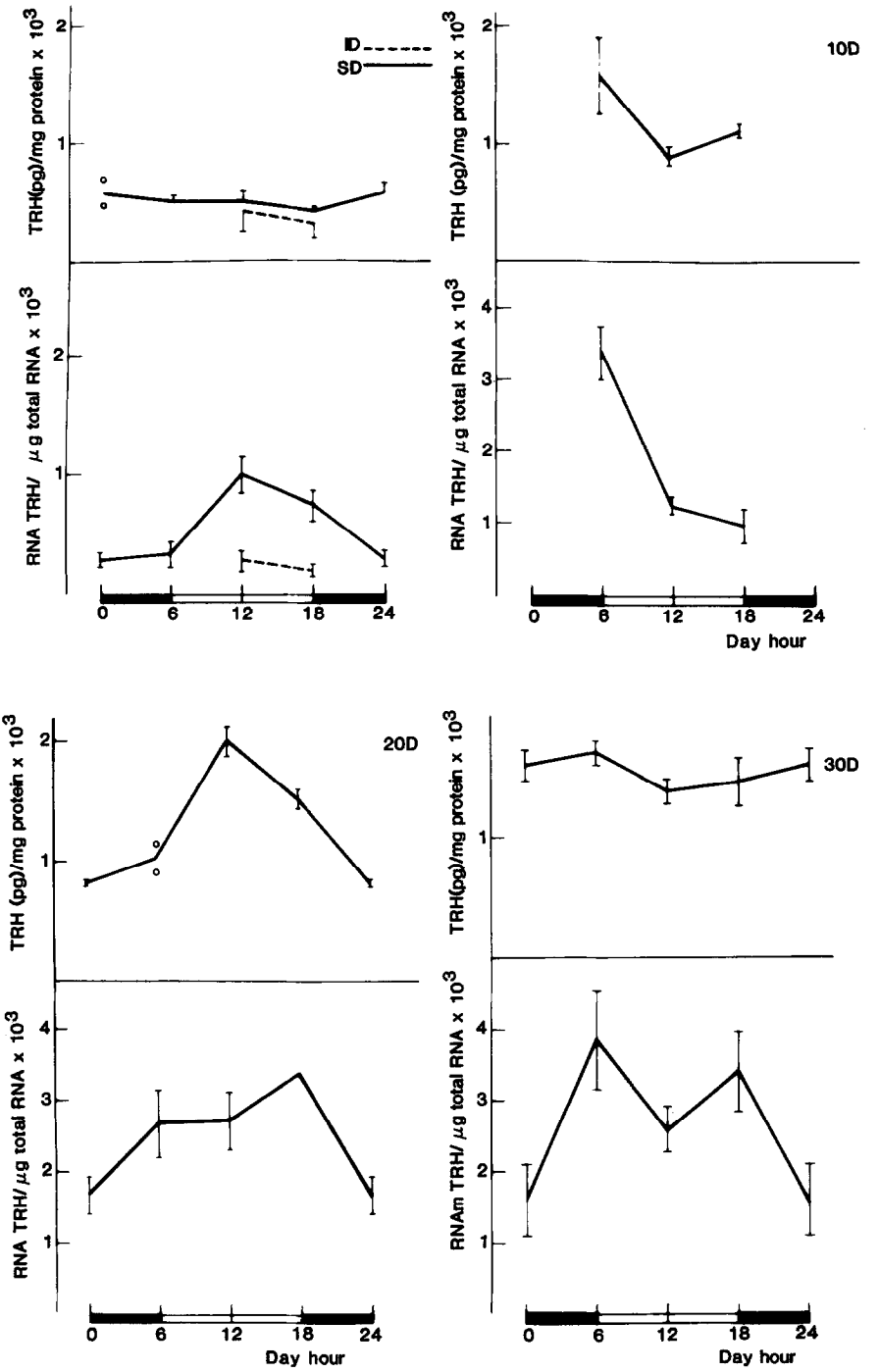

Fig. 3. Development of circadian TRH and TRH mRNA variations. Circadian TRH and TRH mRNA variations were measured at different days after birth. Observe that in most cases TRH and TRH mRNA peaks occur at the same time. Mean ± SEM (n=3).

be adressed to estimate the regulatory role of translation and postranslational events in this system.

We do not know when release is ocurring in the two phenomena analyzed,

therefore, synthesis and release might not be directly coupled. Direct measure of in vivo release is experimentally difficult; however, bare TRH variations during circadian cycle suggest active release at time of synthesis. Accordingly, a recent report (17) and our own observations (18) show that TRH mRNA of paraventricular nucleus (containing most TRH-ergic neurons sending axons to the median eminence) or whole hypothalamus increases in hypothyroid rats whereas median eminence TRH remains unchanged (20).

In addition we observed in in vitro experiments, performed as described (14), that TRH content in hypothalamus or e.g. cervical part of spinal chord do not change despite release. Mean ± SEM percentage of TRH tissue content released in 60min (hypothalamus) or 70 min (cervical part of spinal chord), including a 10 min stimulation period with 56mM KCl, were 9.9 ± 1.8% (n= 8) and 47.8 ± 4.9% (n= 6) respectively. We suspect now that TRH released levels are underestimated due to degradation by pyroglutamate amino peptidase II activity which is 5-fold higher in hypothalamic that spinal chord membranes (21). This suggests that at least ∿50% of total initial content is released after 60 or 70 min incubation from either tissue and therefore a biosynthetic process at the nerve terminal might be coupled to release, i.e. amidation reaction that forms TRH from pglu-his-pro-gly and is activated by ascorbic acid (22); the enzymes involved, could be regulated as reported for MSH acetylation (5). Regulation of intracellular degradation seems unlikely since inhibition of the two soluble intracellular degrading enzymes do not affect endogeneous TRH (23) nor thyroid hormone status affect degrading enzyme activity at hypothalamic level (24). Segerson et al have postulated that other active peptides could be synthesized from the same TRH-precursor (25). Although this might also explain our results since a proportion of TRH mRNA measured could give rise to other active peptides and be regulated differentially, this hypothesis still is speculative.

In conclusion, we propose a regulatory mechanism responsible of establishing TRH levels at nerve terminal working actively after release.

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REFERENCES

1.- Roberts, J.L., Eberwine, J.H. and Gee, C.E. (1985) Symposia on Quantitative Biology Vol. XLIX, 385-391.
2.- Eiden, L.E., Giraud, P., Dave, J.R., Hotchkiss, A.J. and Affolter, H.-U. (1984) Nature 312, 661-663.
3.- Shupnik, M.A., Greenspan, S.L. and Ridgway, E.C. (1986) J. Biol. Chem. 261- 12675-12679.
4.- Shiomi, H., Watson, S.J., Kelsey, J.E. and Akil, H. (1986) Endocrinology 119, 1793-1799.

5.- Millington, W.R., O'Donohue, T.L., Chappell, M.C., Roberts, J.L. and Mueller, G.P. (1986) Endocrinology 118, 2024-2033.
6.- Prasad, C. (1985) In: Handbook of Neurochemistry (Lajtha, A. ed.) Vol. 8. Plenum Publishing Corporation, New York, 175-200.
7.- Lechan, R.M., Wu, P., Jackson, I.M.D., Wolf, H., Cooperman, S., Mandel, G. and Goodman, R.H. (1986) Science 231, 159-161.
8.- Bauer, K. and Kleinkauf, H. (1980) Eur. J. Biochem. 106, 107-117.
9.- Wilk, S. (1986) Life Sciences 39, 1487-1492.
10.- Garat, B., Miranda, J., Charli, J-L. and Joseph-Bravo P. (1985) Neuropeptides 6, 27-40.
11.- Martino, E., Seo, H., Lernmark, A. and Refetoff, S. (1980) Proc. Natl. Acad. Sci. USA 77, 4345-4348.
12.- Martino, E., Bambini, G., Bartalena, L., Aghini-Lombardi, F., Breccia, M. Baschieri, L. and Pinchera, A. (1986) Endocrinology 119, 232-235.
13.- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A laboratory Manual, Cold Spring Harbor Laboratory, New York.
14.- Mendez, M., Joseph-Bravo, P., Cisneros, M., Vargas, M.A. and Charli J-L. (1987) Peptides 8, 291-298.
15.- Gayo, L., Bonet, B., Herranz, A.S., Iglesias, R., Toro, M.J. and Montoya E. (1986) Acta Endocrinologica 112, 7-11.
16.- Lamberton, R.P., Lechan, R.M. and Jackson, I.M.D. (1984) Endocrinology 115, 2400-2405.
17.- Segerson, T.P., Kauer, J., Wolfe, H.C., Mobtaker, H., Wu, P., Jackson, I.M.D. and Lechan, R.M. (1987) Science 238, 78-80.
18.- Covarrubias, L., Uribe, R.M., Mendez, M., Charli, J-L. and Joseph-Bravo, P. (1987) In: Recent Advances in the Biomedical Significance of Thyrotropin Releasing Hormone, Annals of the New York Academy of Sciences, New York (in press).
19.- Kerdelhue, B., Palkovits, M., Karteszi, M. and Reinberg, A. (1981) Brain Research 206, 405-413.
20.- Kardon, F., Marcus, R.J., Winokur, A. and Utiger, R.D. (1977) Endocrinology 100, 1604-1609.
21.- Vargas, M.A., Mendez, M., Cisneros, M., Joseph-Bravo, P. and Charli J-L. (1987) Neuroscience Letters 79, 311-314.
22.- Glembotski, C.C., Manaker, S., Winokur, A. and Gibson, T.R. (1986) J. Neurosc. 6, 1796-1802.
23.- Charli, J-L., Mendez, M., Joseph-Bravo, P. and Wilk, S. (1987) Neuropeptides 9, 373-378.
24.- Ponce, G., Charli, J-L., Pasten, J., Aceves, C. and Joseph-Bravo, P. (1987).
25.- Segerson, T.P., Hoefler, H., Childers, H., Wolfe, H.J., Wu, P., Jackson, I.M.D. and Lechan (1987) Endocrinology 121, 98-107.