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Hypo- and hyperthyroidism affect NEI concentration in discrete brain areas of adult male rats

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ABSTRACT

To date, there has been only one in vitro study of the relationship between neuropeptide EI (NEI) and the hypothalamic–pituitary–thyroid (HPT) axis. To investigate the possible relationship between NEI and the HPT axis, we developed a rat model of hypothyroidism and hyperthyroidism that allows us to determine whether NEI content is altered in selected brain areas after treatment, as well as whether such alterations are related to the time of day. Hypothyroidism and hyperthyroidism, induced in male rats, with 6-propyl-1-thiouracil and L-thyroxine, respectively, were confirmed by determination of triiodothyronine, total thyroxine, and thyrotropin levels. All groups were studied at the morning and the afternoon. In rats with hypothyroidism, NEI concentration, evaluated on postinduction days 7 and 24, was unchanged or slightly elevated on day 7 but was decreased on day 24. In rats with hyperthyroidism, NEI content, which was evaluated after 4 days of L-thyroxine administration, was slightly elevated, principally in the preoptic area in the morning and in the median eminence-arcuate nucleus and pineal gland in the afternoon, the morning and afternoon NEI contents being similar in the controls. These results provide the bases to pursue the study of the interaction between NEI and the HPT axis.

1. Introduction

Neuropeptide glutamic acid-isoleucine-amide (NEI) is a linear, 13-amino acid peptide. It arises from the cleavage of prepro-melanin-concentrating hormone (ppMCH), which also gives rise to melanin-concentrating hormone (MCH) and neuropeptide glycine–glutamic acid [29,32,36]. In rodents and humans, ppMCH is primarily expressed in the incerto-hypothalamic area [5,6,38] and in the lateral hypothalamic area [5], locations from which it is projected widely throughout the central nervous system [3,5,6,36,45]. This broad distribution confers neurotransmitter and neuromodulatory functions on these peptides [3,7,30]. As neurotransmitter, it has been demonstrated that NEI induces grooming behavior and locomotor activity in rats [4,40–43], and also stimulates sexual receptivity in female rats [17]. In terms of neuroendocrine function, it has been shown that NEI is able to induce the release of luteinizing hormone (LH) in vivo [1,2] and in vitro [10]. Studies performed in our laboratory demonstrated that intra-cerebroventricular administration of NEI increases LH serum levels in male rats and in chronically ovariectomized female rats treated with estradiol and low doses of progesterone [1]. Our group proposed various possible pathways by which NEI induces LH secretion, directly or indirectly:

Abbreviations: NEI, neuropeptide glutamic acid-isoleucine-amide; ppMCH, prepro-melanin concentrating hormone; MCH, melanin-concentrating hormone; LH, luteinizing hormone; NEI-ir, NEI-immunereactive; GnRH, gonadotropin-releasing hormone; POA, preoptic area; AVPV, anteroventral periventricular nucleus; ME, median eminence; OVLT, organum vasculosum of the lamina terminalis; HPT, hypothalamic–pituitary–thyroid; TRH, thyrotropin-releasing hormone; TSH, thyrotropin; THs, thyroid hormones; L-T4, L-thyroxine; hypoT, hypothyroidism induction; hyperT, hyperthyroidism induction; T3, triiodothyronine; PVH, paraventricular hypothalamic nucleus; PLH, peduncular part of the lateral hypothalamus; PeFLH, perifornical part of the lateral hypothalamus; ME + Arc, ME and arcuate nucleus; PP, posterior pituitary; Pi, pineal gland; 125I-Tyr NEI, tyrosine radioiodinated NEI.

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by modulation of gonadotropin releasing hormone (GnRH) neurons located in the preoptic area (POA); by GnRH terminals located in the median eminence (ME); by an additive effect involving other neurotransmitters or neurohormones; indirectly, by modulating anteroventral periventricular nucleus (AVPV) neurons [2]. In the only previous study investigating the interaction between NEI and the hypothalamic–pituitary–thyroid (HPT) axis, it was shown that NEI and MCH both inhibit the release of thyrotropin-releasing hormone (TRH) in the medial basal hypothalamus, thus blocking the stimulatory effect that TRH has on thyrotropin (TSH) secretion [21]. It is well known that thyroid hormones (THs) play a fundamental role in differentiation, growth, metabolism, and reproduction [22,50]. In addition, thyroid disorders are responsible of alterations in the normal physiology of the hypothalamic pituitary gonadal axis [9,22,47] and also changes in behavior and locomotor activity [11,16]. In recent years, the regulation of the HPT axis at the brain level has been widely studied [8,13,27,34,35,47].

The aim of the present study is to establish the possible relationship between NEI and the HPT axis, as well as to investigate the effects that thyroid hormones may exert on NEI concentration in various brain areas related to metabolic control and reproduction. The possible consequences derived from NEI changes will be also discussed in this manuscript.

2. Materials and methods

2.1. Animals and treatments

Adult male Wistar rats aged 10–16 weeks and weighing 220–340 g, bred in our laboratory, were maintained on a 14/10-h light/dark cycle in a temperature-controlled environment ($22 \pm 2\,^{\circ}C$) with ad libitum access to standard rat chow and water. The procedures performed in animals were consistent with the standards established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and the American Veterinarian Medical Association Guidelines on Euthanasia. The animals were divided into the following groups:

- Control-AM ($n = 8$–12)—sacrificed in the morning (10:00–12:00).
- Control-PM ($n = 8$–12)—sacrificed in the afternoon (17:00–19:00).
- hypoT-7-AM ($n = 6$–8)—hypothyroid, sacrificed in the morning (10:00–12:00) on post-induction day 7.
- hypoT-7-PM ($n = 6$–8)—hypothyroid, sacrificed in the afternoon (17:00–19:00) on post-induction day 7.
- hypoT-24-AM ($n = 6$–8)—hypothyroid, sacrificed in the morning (10:00–12:00) on post-induction day 24.
- hypoT-24-PM ($n = 6$–8)—hypothyroid, sacrificed in the afternoon (17:00–19:00) on post-induction day 24.
- hyperT-AM ($n = 6$–8)—hyperthyroid, sacrificed in the morning (10:00–12:00) on the day after the administration of the fourth dose of L-thyroxine (L-T4).
- hyperT-PM ($n = 6$–8)—hyperthyroid, sacrificed in the afternoon (17:00–19:00) on the day after the administration of the fourth dose of L-T4.

Hypothyroidism (hypoT) was induced with 6-propyl-1-thiouracil (PTU, Sigma) at a concentration of 0.1 g/l administered in drinking water [18]. Two PTU treatment lengths were used in this study, a short-term induction of hypoT (7 days) and a long-term one (24 days). These two periods were selected based on the results of Kundu et al. [25] where it was demonstrated that short-term hypoT does not modify brain THs levels, concluding that more than 20 days of treatment are necessary to induce a reduction in THs concentration at this level. This mechanism is called "brain thyroid hormone homeostasis" [25]. Therefore, we hypothesized that if NEI content is modulated by changes in brain THs concentrations, it is possible that NEI levels may not change during the brain thyroid hormone homeostasis period, but that changes may be observed with a longer period of hypoT induction (24 days).

Hyperthyroidism (hyperT) was induced with L-thyroxine (L-T4, Sigma), 250 μg/kg body weight administrated subcutaneously for 4 days [39].

2.2. Samples and preparation of tissue extracts

Animals were sacrificed by decapitation. Trunk blood was immediately collected and stored at 4 °C until processing. Prior to extraction of the brain, the pineal gland (Pi) was obtained. Each brain was removed and immediately placed on an aluminum plate at 4 °C for the cutting of specific coronal rat brain slices in a rat brain matrix (RBM 4000C; ASI Instruments, Inc., Warren, MI, USA). The posterior pituitary (PP) gland was also harvested. The Pi and PP were both placed separately in 100 μl of 0.06 N HCl and stored at 4 °C until processing. Each brain was serially sectioned in order to obtain five 1-mm coronal slices approximately; the final part of the olfactory tubercles and the beginning of the optic chiasm were used as limits anterior to bregma (approximately 0.12 mm), and the final part of the ME was used as limit posterior to bregma (approximately −3.48 mm) [33]. For obtaining each slice Gillette blades were used. After cutting, each brain slice was put on a cold glass plate and carefully spread out. All proceedings were done at 4 °C. The areas of interest were grouped as follows: from slice 1, the OVLT + AVPV; from slices 1 and 2, the lateral and medial POA; from slices 2 and 3, periventricular nucleus; from slice 3, the paraventricular hypothalamic nucleus (PVH); from slices 3 and 4, the peduncular part of the lateral hypothalamus (PLH); from slice 4, the perifornical part of the lateral hypothalamus (PeFLH); from slices 4 and 5, the ME and arcuate nucleus (ME + Arc). The tissue was observed under a magnifying glass and brain areas were taken according to Paxinos and Watson [33]. Areas were dissected one per one with the sharp end of a needle (23-gage × 1 in.; Terumo, Tokyo, Japan). For peptide extraction each brain sample was homogenized in 100 μl of 0.06 N HCl and boiled for 10 min. The homogenates were centrifuged at 3000 rpm at 4 °C to separate solid debris. Subsequently, the supernatants were collected, the pellets were resuspended in 100 μl of 0.06 N HCl and a second centrifugation was performed under the same conditions. The first and second supernatants were combined and stored at −20 °C for later radioimmunoassay (RIA). In the pellets, we determined proteins by Bradford assay [24]. Serum was obtained from troncal blood after centrifugation at 3000 rpm for 20 min at 4 °C and was used in the RIA to determine TSH, total T3, total thyroxine (T4), LH, and testosterone.

2.3. NEI antibody

Rat NEI antibody raised in rabbit (Code PBL #237) was kindly provided by Drs. PE Sawchenko and JC Bittencourt. NEI RIA has previously been shown to be highly specific [10,19,48,49]. The NEI antisera can identify amidated NEI, NEI-G, and NEI-GR forms but cross reacts poorly with other pro-MCH derivates and the precursor itself [19,48]. Therefore the NEI-IR concentrations are a reliable indicator of the levels of the processed peptide [49].

2.4. NEI assay

Prior to the RIA determination of NEI concentration, each brain sample was treated with 50 μl of 250 mM phosphate buffer to achieve pH 7. NEI determination in the samples was performed by RIA as previously described [32]. In brief, Tyr-NEI (Bachem, Torrance, CA, USA) was radio-iodinated with chloramine T and

Table 1
Circulating hormone levels of TSH, T3, T4, LH and T in the groups of control and treated male rats.

	TSH ng/ml	T3 ng/dl	T4 μg/dl	LH ng/ml	Testosterone ng/ml
Control-AM	1.33 ± 0.35a	85.3 ± 4.57a	3.53 ± 0.16a	1.04 ± 0.19b	0.88 ± 0.35a
hypoT-7-AM	7.60 ± 0.61a***	22.54 ± 1.18a***	1.41 ± 0.31b**	1.68 ± 0.25a	1.69 ± 0.31a
hypoT-24-AM	ND	23.76 ± 4.00a***	0.27 ± 0.11a***	ND	1.21 ± 0.36a
hyperT-AM	0.20 ± 0.03a**	237.50 ± 18.28a***	25.24 ± 3.58a***	1.81 ± 0.25a	0.65 ± 0.19a
Control-PM	0.65 ± 0.19a	70.2 ± 4.02a	3.71 ± 0.28a	2.84 ± 0.44a	1.13 ± 0.22a
hypoT-7-PM	7.77 ± 0.67a***	25.4 ± 2.42a***	0.43 ± 0.14a***	1.88 ± 0.44a	0.78 ± 0.20a
hypoT-24-PM	ND	23.00 ± 2.53a***	0.10 ± 0.03a***	ND	0.65 ± 0.17a
hyperT-PM	0.46 ± 0.12a	195.38 ± 23.36a***	17.25 ± 2.79a***	2.17 ± 0.33a	1.08 ± 0.43a

Each value represents mean ± SEM.
ND: not determined.
Letters represent the comparison of each group under study at AM and PM, a represents no significantly differences. b represents significantly differencespobserved in T4 levels in hypoT-7-AM vs. hypoT-7-PM, b: $p < 0.01$; and in LH levels in control-AM vs. control-PM, b: $p < 0.01$.
** Comparison of CTR vs. treated groups at the same time studied: $p < 0.01$.
*** Comparison of CTR vs. treated groups at the same time studied: $p < 0.001$.

separated from free iodine in a Sephadex G25 column (Pharmacia, Uppsala, Sweden) with 0.2 M acetic acid as elution buffer. The RIA was performed by incubating 50-μl aliquots of each sample, with appropriately diluted NEI antibody and 125I-Tyr NEI (9000–10000 cpm), in a finl volume of 300 μl in PBS, 0.1% sodium azide, 0.1% BSA, 0.33% normal rabbit serum, and 33 mM EDTA, for 20–24 h at 4 °C. Bound and free 125I-Tyr NEI were separated by addition of 50 μl of PBS with goat anti-rabbit antibody (1:15) and incubated for 60 min, after which 25% polyethylene glycol 6000 was added and the samples were centrifuged at 3000 rpm for 30 min at 4 °C. The supernatants were then discarded, and the pellets were counted in a gamma counter. Assay sensibility was 1 pg/tube; intra- and inter-assay coefficients of variation were <10%. The final values for NEI content in the various brain areas were expressed as ng NEI/mg protein.

2.5. Hormone determinations

Circulating TSH and LH were measured by double antibody radioimmunoassay, using materials provided by Dr. AF Parlow and the NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA). The hormones were radioiodinated using the Chloramine T method and purified by passage through Sephadex G75. The results were expressed in terms of the rat TSH and LH RP-3 standard preparations. Assay sensitivities were 0.5 μg/l serum and the intra-assay coefficients of variation were less than 10% for both RIAs. All the samples were measured on the same assay by duplicate. T3 and T4 were determined using commercial RIA kits for total hormones (Coat-a-Count; Siemens Medical Solutions, Los Angeles, CA, USA). Testosterone was measured in acidified serum (5 μl of 0.1 N HCl/50 μl of serum sample) with a commercial RIA kit (KIP1709; DIAsource ImmunoAssays S.A., Nivelles, Belgium).

2.6. Statistical analysis

The statistical analysis was performed using two-way ANOVA with Bonferroni post-test. When the data did not show a normal distribution, the values were log-transformed to ensure variance homogeneity. The differences between means were considered significant at $p < 0.05$.

3. Results

3.1. Circulating hormone levels

Hypothyroidism or hyperthyroidism (Table 1) modified circulating hormone levels. As expected, TSH values were elevated by hypoT at both times of day and lowered by hyperT, although significant differences were found only between the control-AM and hyperT-AM groups. In contrast, circulating T3 and T4, compared with controls were significantly higher in both hyperT groups and significantly decreased in the hypoT groups. In the hypoT rats there were no significant differences between treatments and time of day for T3, but T4 levels were lower in the 24 days treatment group compared with the 7 days group, and also, T4 values were higher in the morning compared with the afternoon values. In controls, LH values were higher in the afternoon compared with morning values, but this difference was abolished in the treated groups, because in these latter groups LH values tended to be higher in the morning and lower in the afternoon. No differences were observed among the groups in testosterone levels.

3.2. NEI concentration in the lateral hypothalamic area

In the PLH, the morning and afternoon NEI concentrations were similar in all groups. The NEI levels were highest in the hypoT-7 groups and lowest in the hypoT-24 groups, although neither was significantly different in comparison with the controls (Fig. 1A). No differences were observed between the hyperT and control groups.

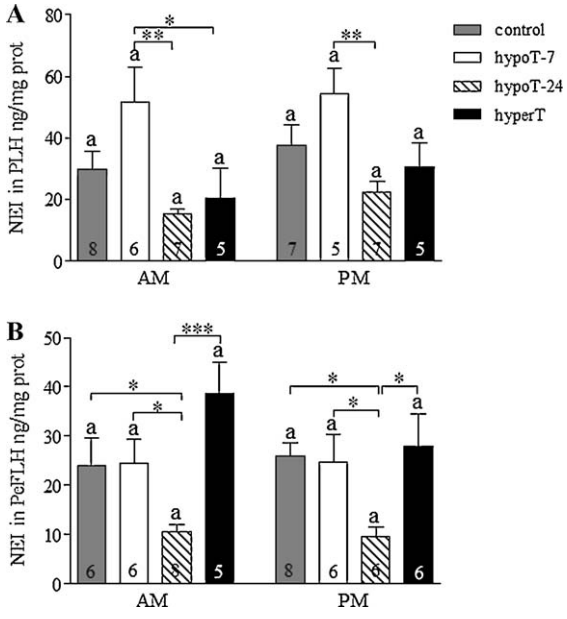

Fig. 1. NEI content in brain areas related to peptide synthesis. (A) In the peduncular part of the lateral hypothalamus (PLH) and (B) in the perifornical part of the lateral hypothalamus (PeFLH): *comparison among the various groups studied at the same time; a represents the comparison of each group under study—AM vs. PM. Each column represents mean ± SEM, total number of samples is shown at the base of each column. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

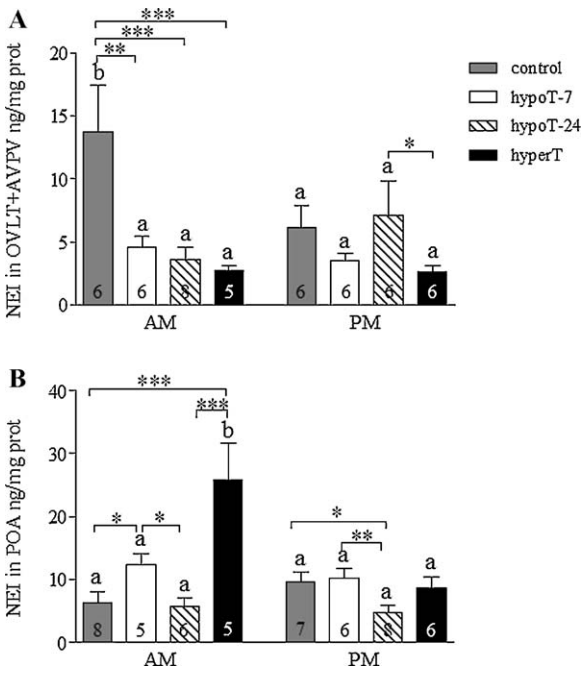

Fig. 2. NEI content in brain areas related to reproduction. (A) In the organum vasculosum of the lamina terminalis–anteroventral periventricular nucleus (OVLT + AVPV) and (B) in the preoptic area (POA): *comparison among the various groups studied at the same time; letters represent the comparison of each group under study—AM vs. PM. Each column represents mean ± SEM, total number of samples is shown at the base of each column. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. In panel A: $^{b}p < 0.05$; in panel B: $^{b}p < 0.01$.

In the PeFLH, the morning and afternoon NEI concentrations were similar in the control; and in the hypoT-7 groups and hyperT had no significant effect. However, 24 days hypoT reduced NEI levels at both time points measured (Fig. 1B).

3.3. NEI concentration in brain areas with known NEI and hypophysiotrophic TRH fibers

NEI concentration in the OVLT + AVPV, of control rats was higher in the morning than in the afternoon, and all the treatments decreased significantly NEI levels in the morning, but had no effect on the low values observed in the afternoon (Fig. 2A).

In the POA, there were no differences between morning and afternoon values in all groups except for the hyperT rats, where the morning values were significantly higher than the afternoon ones. In the morning, NEI levels were significantly higher than controls in the hypoT-7 and hyperT groups, while in the afternoon the only difference observed was a significant reduction in NEI levels in the hypoT-24 group (Fig. 2B).

In the periventricular nucleus, there were no significant differences among the groups (Fig. 3A).

In the ME + Arc, the morning and afternoon NEI concentrations were comparable within groups and there were no differences among the groups in the morning. In contrast, in the afternoon, NEI levels were lowered in the hypoT-24 group and increased in the hyperT group when compared with controls (Fig. 3B).

3.4. NEI concentration in the paraventricular hypothalamic nucleus

In the PVH, there were no differences between morning and afternoon NEI concentrations within groups, the hypoT-7 and hyperT groups did not differ from the controls, and only the hypoT-24 group had significantly lower values (Fig. 4).

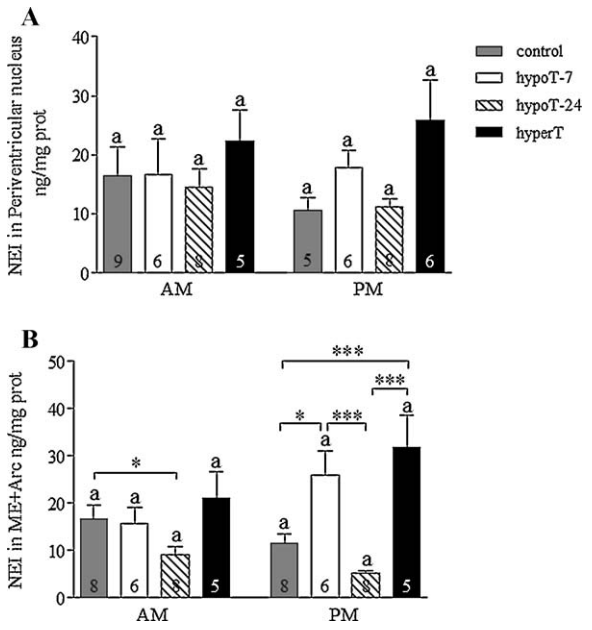

Fig. 3. NEI content in brain areas related to reproduction. (A) In the periventricular nucleus and (B) in the median eminence-arcuate nucleus (ME + Arc): *comparison among the various groups studied at the same time; a represents the comparison of each group under study—AM vs. PM. Each column represents mean ± SEM, total number of samples is shown at the base of each column. $^{*}p < 0.05$ and $^{***}p < 0.001$.

3.5. NEI concentration in posterior pituitary and pineal gland

In the PP, there were no significant differences among groups or time points (Fig. 5A).

In the Pi, NEI concentration did not differ between the control-AM and control-PM groups, but it was different between hypoT-AM and hypoT-PM groups, with a tendency to decrease in hypoT-7 and to increase in the hypoT-24 in the afternoon. Afternoon NEI concentration in the Pi was higher in the hyperT group (Fig. 5B).

4. Discussion

The potential regulatory effect of thyroid hormones on NEI has not been previously studied. Determining the changes in NEI concentration secondary to hypothyroidism or hyperthyroidism represents a means of investigating this interaction.

In our control animals, except in OVLT + AVPV, NEI levels were similar at the times studied in all the areas we explored. This suggests that NEI synthesis and release in these areas is not subject to circadian variations, under normal conditions, at least during the lights on period. In a previous study evaluating 24-h expression

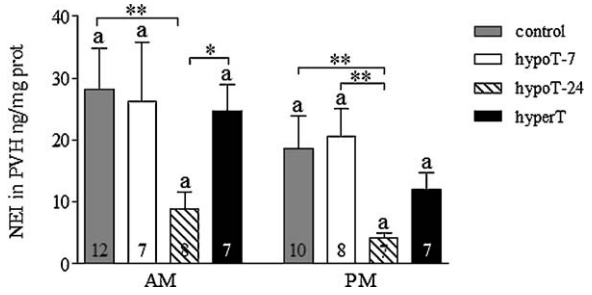

Fig. 4. NEI content in the paraventricular nucleus. In the paraventricular hypothalamic nucleus (PVH): *comparison among the various groups studied at the same time; a represents the comparison of each group under study—AM vs. PM. Each column represents mean ± SEM, total number of samples is shown at the base of each column. $^{*}p < 0.05$ and $^{**}p < 0.01$.

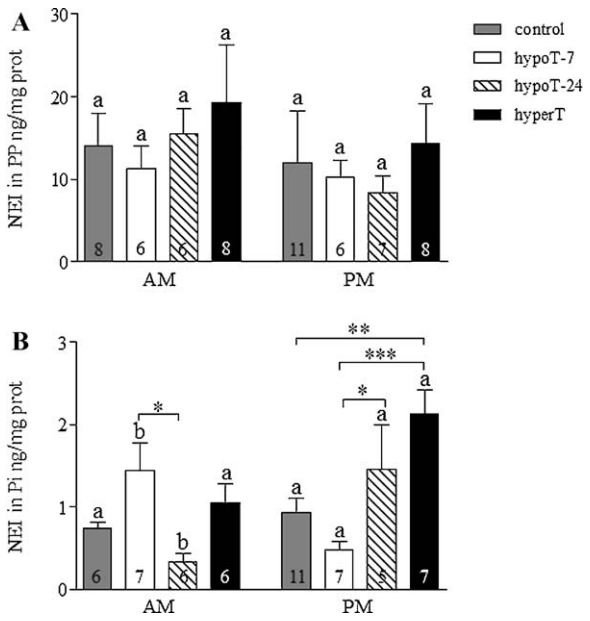

Fig. 5. NEI content in the posterior pituitary (PP) and pineal gland (Pi). (A) PP and (B) Pi: *comparison among the various groups studied at the same time; a represents the comparison of each group under study—AM vs. PM. Each column represents mean ± SEM, total number of samples is shown at the base of each column. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. In panel B: hypoT-7-AM vs. hypoT-7-PM and hypoT-24-AM vs. hypoT-24-PM, $^{b}p < 0.05$.

of various genes in the mouse hypothalamus (12/12-h light/dark cycle), the expression of MCH was found to remain stable between 10:00 and 22:00 [46]. These results support our findings suggesting that, under normal conditions, peptide levels do not change over the course of the day.

Different pattern was observed in the control OVLT + AVPV where NEI levels changed between the morning and the afternoon. We found higher NEI concentration in the morning and a decreased in the afternoon. Also in this area all treatments induced a reduction in NEI concentration at morning. Further studies will be necessary to understand the mechanisms involve in NEI regulation in these areas.

Short-term hypothyroidism (7 days) had a very small effect on NEI level in most of the areas of interest, only the morning levels in the POA and the afternoon levels in the ME + Arc were significantly higher in the hypoT-7 group, while OVLT + AVPV NEI concentration in the morning were lower. Long-term hypothyroidism (24 days), however, had a greater effect, causing morning and afternoon reductions in the PeFLH and PVH, reduction in the OVLT + AVPV and ME + Arc only in the morning, and reductions in the POA only in the afternoon. There is a close relationship between the thyroid hormone levels found in the brain by Kundu et al. [26] and the NEI contents observed in the present study. Thus, the short term treatment not have been able to lower thyroid hormone levels in the studied areas, and thus had a very slight effect on NEI concentrations, while after 24 days of treatment, cerebral thyroid hormone levels were lowered and thus were able to induce changes in NEI levels. These results suggest that NEI synthesis and release are modulated by T3 levels in the central nervous system.

In the PVH, NEI concentrations were reduced in the long term hypothyroid rats. Since Kennedy et al. [21] have shown that NEI inhibits TRH release, the low NEI levels in the PVH may have contributed to increase TRH levels as a response to the hypothyroid state, although this effect may be probably minor compared to thyroid hormones feedback acting directly on TRH neurons.

There are few recent studies on the effects of hyperthyroidism on the adult brain and most of these have focused on the effects of thyroid hormones on the regulation of TRH producing neurons [12,14,31]. In the present study, hyperthyroidism altered NEI concentrations only in the POA in the morning, and in the ME + Arc and Pi in the afternoon. The pattern observed in the POA and in the ME + Arc in the afternoon suggests that NEI is released from the POA and later accumulates in the ME + Arc.

The Pi is also involved in neuroendocrine control [15,28] and reproduction [37]. Although baseline levels of NEI are 10–15-fold lower than in other areas studied, and this gland may receive MCH-related peptides from the blood in addition to direct innervations, we observed changes secondary to hyperthyroid status induction. The present data is indicative that NEI may play a role in Pi gland function. Further studies should be done to clarify the role of NEI in this gland.

All of the brain areas evaluated in the present study are involved in neuroendocrine regulation, hormone release, and reproduction [2,20,23,44]. Although the relationship between NEI and reproduction in male rats has yet to be studied in depth, it has been widely investigated in females. In male rats, intra-cerebroventricular NEI injection has been shown to induce higher LH serum levels [1]. Studies conducted in female rats have demonstrated that NEI stimulates sexual behavior when injected into the medial POA [17]. It has also been shown that, in female rats, NEI induces higher LH serum levels and is closely related to GnRH fibers in various hypothalamic areas [1,2]. Although there are marked sex-related differences, we can suggest that NEI is also related to reproduction in males. All of the brain areas in which NEI has been studied are also related to male rat reproduction [20]. If we assume that thyroid hormones have direct effects on reproduction and behavior [9,11,16,22,47], and that NEI is also implicated in these effects, it is likely that some of the alterations observed in hypothyroidism and hyperthyroidism may be also mediated by NEI among others neuroendocrine modulators. Therefore, thyroid hormone-induced changes in NEI content could have important consequences for the reproductive function of NEI.

5. Conclusion

Hypothyroidism and hyperthyroidism both influence NEI concentration in discrete brain areas related to neuroendocrine control of reproduction. There is a direct relationship between NEI concentrations and thyroid hormone levels being lower in hypothyroidism and higher in hyperthyroidism.

On the basis of these results, we can propose that thyroid hormones have a differential regulation of NEI levels in several brain areas probably through a modulation of its synthesis and release. However, further studies are needed to elucidate the different mechanisms involved in the interaction between NEI and the HPT axis.

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