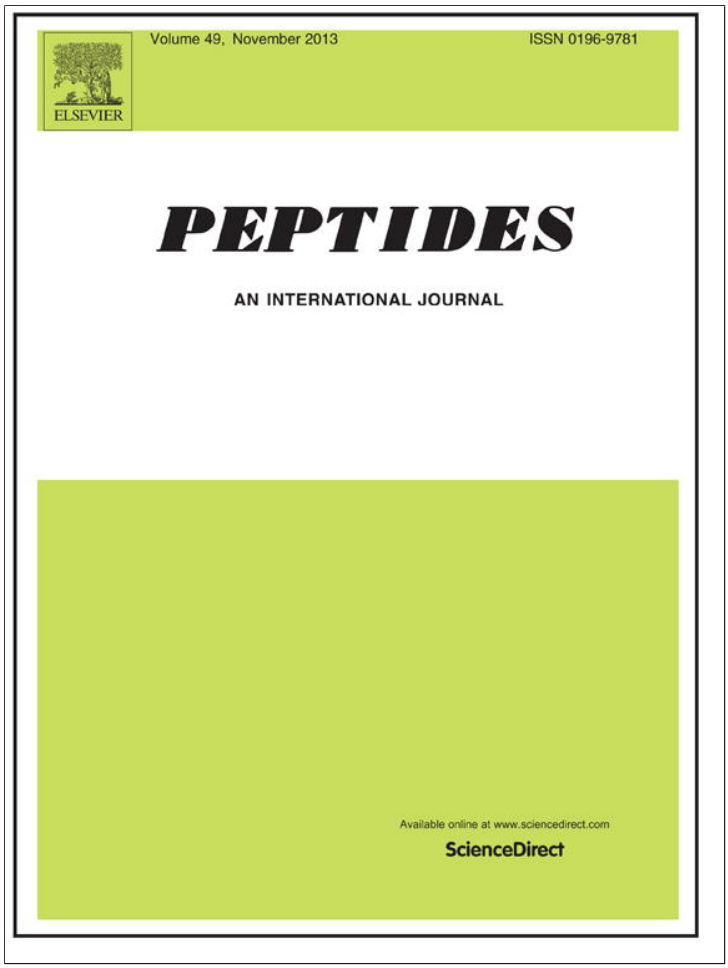

Peptides 49 (2013) 74–80

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Effects of thyroid status on NEI concentration in specific brain areas related to reproduction during the estrous cycle

Carolina Ayala[a,b], Gisela Erika Pennacchio[c], Marta Soaje[c,d], Norma Beatriz Carreño[c], Jakson Cioni Bittencourt[e], Graciela Alma Jahn[c], María Ester Celis[a], Susana Ruth Valdez[c,f,*]

[a] Laboratorio de Ciencias Fisiológicas, Cátedra de Bacteriología y Virología Médicas, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, CP 5000 Córdoba, Argentina
[b] Sección de Desarrollo Cerebral Perinatal (SPBD), Instituto de Histología y Embriología Mendoza (IHEM-CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Parque General San Martín, CP 5500 Mendoza, Argentina
[c] Laboratorio de Reproducción y Lactancia, Instituto de Medicina y Biología Experimental de Cuyo-Consejo Nacional de Investigaciones Científicas y Técnicas (IMBECU-CONICET), CCT-CONICET-Mendoza, CP 5500 Mendoza, Argentina
[d] Departamento de Morfofisiología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Parque General San Martín, CP 5500 Mendoza, Argentina
[e] Laboratory of Chemical Neuroanatomy, Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, PC 05508-000 São Paulo, Brazil
[f] Instituto de Ciencias Básicas (ICB), Universidad Nacional de Cuyo, Parque General San Martín, CP 5500 Mendoza, Argentina

ARTICLE INFO

Article history:
Received 14 June 2013
Received in revised form 21 August 2013
Accepted 21 August 2013
Available online 9 September 2013

Keywords:
Neuropeptide
glutamic-acid-isoleucine-amide (NEI)
Hypothalamus
Hypothyroidism
Hyperthyroidism
Estrous cycle
Reproduction

ABSTRACT

We previously showed that short-term hypo- and hyperthyroidism induce changes in neuropeptide glutamic-acid-isoleucine-amide (NEI) concentrations in discrete brain areas in male rats. To investigate the possible effects of hypo- and hyperthyroidism on NEI concentrations mainly in hypothalamic areas related to reproduction and behavior, female rats were sacrificed at different days of the estrous cycle. Circulating luteinizing hormone (LH), estradiol and progesterone concentrations were measured in control, hypothyroid (hypoT, treated with PTU during 7–9 days) and hyperthyroid (hyperT, L-T_4 during 4–7 days) animals. Both treatments blunted the LH surge. Hypo- and hyperthyroidism increased estradiol concentrations during proestrus afternoon (P-PM), although hypoT rats showed lower values compared to control during proestrus morning (P-AM). Progesterone levels were higher in all groups at P-PM and in the hyperT during diestrus morning (D_2). NEI concentrations were lower in hypoT rats during the estrous cycle except in estrus (E) in the peduncular part of the lateral hypothalamus (PLH). They were also reduced by both treatments in the perifornical part of the lateral hypothalamus (PeFLH) during P-PM. Hypothyroidism led to higher NEI concentrations during P-PM in the organum vasculosum of the lamina terminalis and anteroventral periventricular nucleus (OVLT + AVPV). The present results indicate that NEI concentration is regulated in a complex manner by hypo- and hyperthyroidism in the different areas studied, suggesting a correlation between NEI values and the variations of gonadal steroid levels during estrous cycle. These changes could be, in part, responsible for the alterations observed in the hypothalamic–pituitary–gonadal axis in these pathologies.

Abbreviations: NEI, Neuropeptide glutamic-acid-isoleucine-amide; ppMCH, pre-pro-melanin concentrating hormone; LH, luteinizing hormone; HPT, hypothalamic–pituitary–thyroid axis; HPG, hypothalamic–pituitary–gonadal axis; OVLT, organum vasculosum of the lamina terminalis; AVPV, anteroventral periventricular nucleus; IHy, incerto-hypothalamic area; PLH, peduncular part of the lateral hypothalamus; PeFLH, perifornical part of the lateral hypothalamus; ME, median eminence; ME+Arc, ME and arcuate nucleus; PP, posterior pituitary; 125I-Tyr NEI, tyrosine radioiodinated NEI; TRH, thyrotropin-releasing hormone; TSH, thyrotropin; THs, thyroid hormones; L-T_4, L-thyroxine; T_3, triiodothyronine; hypoT, hypothyroidism induction; hyperT, hyperthyroidism induction; E, estrus; P, proestrus; D_2, second day of diestrus; GnRH, gonadotropin releasing hormone; MPA, medial preoptic area; D2, type 2 iodothyronine deiodinase.

* Corresponding author at: Laboratorio de Reproducción y Lactancia, Instituto de Medicina y Biología Experimental de Cuyo-Consejo Nacional de Investigaciones Científicas y Técnicas (IMBECU-CONICET), CCT-CONICET-Mendoza, CP 5500 Mendoza, Argentina. Tel.: +54 2615244156; fax: +54 2615244001.
E-mail addresses: caro.ayala.arg@gmail.com (C. Ayala), gpennacchio@mendoza-conicet.gob.ar (G.E. Pennacchio), msoaje@mendoza-conicet.gob.ar (M. Soaje), nkarrenio@yahoo.com (N.B. Carreño), jcbitten@icb.usp.br (J.C. Bittencourt), gjahn@mendoza-conicet.gob.ar (G.A. Jahn), mefisiol2000@yahoo.com.ar (M.E. Celis), svaldez@mendoza-conicet.gov.ar, valdezsusa@gmail.com (S.R. Valdez).

0196-9781/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.peptides.2013.08.016

1. Introduction

NEI is a 13 amino-acid peptide derived from pre-pro-melanin-concentrating hormone (ppMCH) [44,46,51], widely distributed throughout the central nervous system (CNS) [5,9,10,61]. Previous studies showed that NEI plays different roles in the rat CNS, such as inducing grooming behavior and locomotor activity [7,53–56]. It also participates in the regulation of reproduction, acting as a neurotransmitter stimulating sexual receptivity in females [18] and as a neuromodulator inducing luteinizing hormone (LH) release in vivo [2,3] and in vitro [15]. NEI has also been shown to act on the hypothalamic–pituitary–thyroid axis (HPT axis), inhibiting hypothalamic TRH release [31]. Furthermore, our previous work done in male rats showed that hypo- and hyperthyroidism induce changes in NEI concentrations in discrete hypothalamic areas related to the reproductive axis and in the posterior pituitary (PP), suggesting that this peptide may mediate some of the effects of thyroid hormones on reproduction [4]. Thyroid hormones regulate the normal function of the reproductive system, and hypo- and hyperthyroidism induce alterations on the hypothalamic–pituitary–gonadal axis (HPG axis) in humans [32] and animals [17,20–22,28]. Previous studies in adult cycling female rats have shown that hypothyroidism influences normal follicular maturation of the ovary and gonadotropin secretion, resulting in irregular estrous cycles [24], reduces fertility, and causes spontaneous consecutive pseudopregnancies [21,43]. Hyperthyroidism induced by daily doses of L-thyroxine (L-T_4) produces irregular estrous cycles and alterations in the secretion of preovulatory hormones [17,28].

The aim of the present study was to determine the changes in NEI concentration during the estrous cycle in some hypothalamic areas related to the control of reproduction and sexual behaviors where NEI fibers and cells are located in female rats. We also sought to characterize the effects of hypo- and hyperthyroidism on these patterns, correlating them with the alterations in the HPG axis produced by the abnormal thyroid states. As NEI and thyroid hormones are both involved in the regulation of the HPG axis and in the control of behaviors, such as sexual behavior, our working hypothesis was that an interaction between NEI and thyroid hormones may be involved in the regulation of the HPG axis. The possible mechanism of this relationship and its consequences will be also discussed in this manuscript.

2. Materials and methods

2.1. Animals

Adult Wistar female rats with 4-day regular cycles, aged 10–14 weeks and weighing 180–220 g were used. Animals were bred in our laboratory and maintained on a 14/10-h light/dark cycle (lights on at 06.00 h) in a temperature-controlled environment ($22 \pm 2\,^{\circ}C$) with ad libitum access to standard rat chow and water. Animal maintenance and handling were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and the American Veterinarian Medical Association Guidelines on Euthanasia.

2.1.1. Identification of estrous cycle phases

To identify estrous cycle and to habituate rats to the procedure, vaginal smears were collected using a plastic pipette with NaCl 0.9% during 8 days at 10 h and placed on glass slides. The smears were immediately analyzed under a microscope and the identification of the phases was done according to the predominant cell type, i.e. proestrus (P): smear predominated by nucleated round epithelial cells, estrus (E): smear predominated by cornified epithelial cells and second day of diestrus (D_2): smear with predominance of leucocytes [41]. Only rats with two consecutive normal estrous cycles were selected for the study, and only individuals in P, E, D_2 were used.

2.1.2. Treatments

Induction of hypothyroidism or hyperthyroidism was started at the E day in all studied animals. Hypothyroidism was induced with 6-propyl-2-thiouracil (PTU, Sigma Chemical Co., St. Louis, USA) at a concentration of .1 g/l administered in drinking water [22] during 7–9 days, while hyperthyroidism was induced with L-thyroxine (L-T_4, Sigma Chemical Co., St. Louis, USA) 250 μg/kg administrated subcutaneously [4] for 4–7 days. Animals were divided into 6–8 per group of controls, hypothyroid (hypoT) and hyperthyroid (hyperT) rats, and sacrificed as follows: between 10 and 12 h in P (P-AM), E and D_2 and between 17 and 19 h in P (P-PM).

2.2. Samples and preparation of tissue extracts

Animals were sacrificed by decapitation. Trunk blood was immediately collected and stored in an ice bath at 0–4 °C until processing. Each brain was removed and immediately placed on an ice cold aluminum matrix for the cutting of specific coronal rat brain slices (RBM 4000C; ASI Instruments, Inc., Warren, MI, USA). The posterior pituitary (PP) gland was also harvested, placed in 100 μl of .06 N HCl and stored in an ice bath at 0–4 °C until processing. Each brain was serially sectioned in order to obtain five .7–1 mm coronal slices; between the final part of the olfactory tubercles and the beginning of the optic chiasm (approximately .1 mm anterior to Bregma), and the final part of the median eminence (ME, approximately −3.5 mm posterior to Bregma) [49]. Gillette razor blades were used to obtain each slice. After cutting, each brain slice was put on an ice cold glass plate and carefully spread out. All proceedings were done at 0–4 °C. Each slice was observed under a magnifying glass and brain areas were identified according to Paxinos and Watson [49] and microdissected one per one with the sharp end of a needle (23-gauge x1 in.; Terumo, Tokyo, Japan) and always by the same specialized operator (Dr C. Ayala). The brain areas of interest were taken and grouped as follows: from slice 1 the organum vasculosum of the lamina terminalis and anteroventral periventricular nucleus (OVLT + AVPV); from slices 3 and 4, the peduncular part of the lateral hypothalamus (PLH) and the incerto-hypothalamic area (IHy); from slice 4, the perifornical part of the lateral hypothalamus (PeFLH); and from slices 4 and 5, the ME and arcuate nucleus (ME + Arc).

For peptide extraction each brain sample was homogenized in 100 μl of .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 .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). Proteins were determined in the pellets by Bradford assay [33]. Serum was obtained from troncal blood after centrifugation at 3000 rpm for 20 min and stored at −20 °C until LH, estradiol, progesterone, TSH, T_3 and total T_4 determination by RIA.

2.3. NEI assay

Prior to the RIA determination of NEI concentration, 50 μl of 250 mM phosphate buffer were added to each brain area homogenate in order to achieve pH 7. NEI determination in the samples was performed by RIA as previously described [4,47]. Briefly, Tyr-NEI (Bachem, Torrance, CA, USA) was radio-iodinated with chloramine T and separated from free iodine in a Sephadex G25 column (Pharmacia, Uppsala, Sweden) with .2 M acetic acid as elution buffer. The RIA was performed by incubating 50 μl

aliquots of each sample with appropriately diluted (to obtain 25–30% Bo/T, non-specific binding was always below 3% of total counts) NEI antibody (Code PBL#237, kindly provided by Drs. PE Sawchenko and JC Bittencourt, polyclonal antiserum generated in rabbits [10]) and ^{125}I-Tyr NEI (9000–10,000 cpm), in a final volume of 300 μl in PBS, .1% sodium azide, .1% BSA, .33% normal rabbit serum, and 33 mM EDTA, for 20–24 h at 4 °C. Bound and free ^{125}I-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 sensitivity was 1 pg/tube; intra and inter-assay coefficients of variation were <10%. The final values for NEI concentrations in the various brain areas were expressed as ng NEI/mg protein.

2.4. Hormone determinations

LH and TSH levels were determined by RIA as previously described [4]. Briefly, TSH and LH were radio-iodinated using the chloramine T method and purified by passage through Sephadex G75. The results were expressed in terms of rat TSH RP-3 and LH RP-3 standard preparation. Assay sensitivity was .5 μg/l serum and the inter- and intra-assay coefficients of variation were <10%. Estradiol and progesterone were determined using commercial RIA kits for total hormones (estradiol: Coat-a-Count; Siemens Medical Solutions, Los Angeles, CA, USA; progesterone: RIA Coated tube, IM1188, Immunotech®, Beckman Coulter Company, France). Total T_3 and T_4 were determined by commercial RIA kits in the P-AM samples to confirm the effectiveness of the treatments (Coat-a-Count; Siemens Medical Solutions, Los Angeles, CA, USA).

2.5. Statistical analysis

Statistical analyses were performed using two-way ANOVA with Bonferroni post hoc test to compare any two individual means. Data not showing normal distribution were log-transformed to ensure variance homogeneity. Differences between means were considered significant at $p < 0.05$.

3. Results

3.1. Serum hormone levels

LH levels were highest during P-PM in the control group, which corresponds to the expected LH surge at this time of P. HypoT and hyperT showed significantly lower levels than controls during P-PM (Fig. 1A).

Estradiol levels in control rats peaked during P-AM and declined afterwards. In the hypoT group the peak was delayed to P-PM, while animals of the hyperT group peaked at P-AM and the values remained elevated at P-PM (Fig. 1B).

Progesterone levels were highest in P-PM in all studied groups when compared with the other time points, with the exception of hyperT that also showed high levels during D_2 (Fig. 1C).

Circulating TSH levels (Table 1) and T_3 and T_4 levels in P-AM (Table 2) confirmed the effectiveness of the treatments.

3.2. NEI concentration in brain areas related to peptide synthesis

There was no change in NEI concentration in the IHy along the estrous cycle in control rats; hypoT rats had significantly elevated values on D_2, while hyperT rats had elevated values on D_2 and P-AM when compared with the control groups (Fig. 2).

In the PLH, no differences were found in NEI content of control groups at any time of the estrous cycle. NEI content of the hyperT rats was similar to controls at all times studied. However, lower NEI concentrations were observed in this area in the hypoT on D_2, P-AM and P-PM, while in E NEI concentration returned to levels similar to controls (Fig. 3).

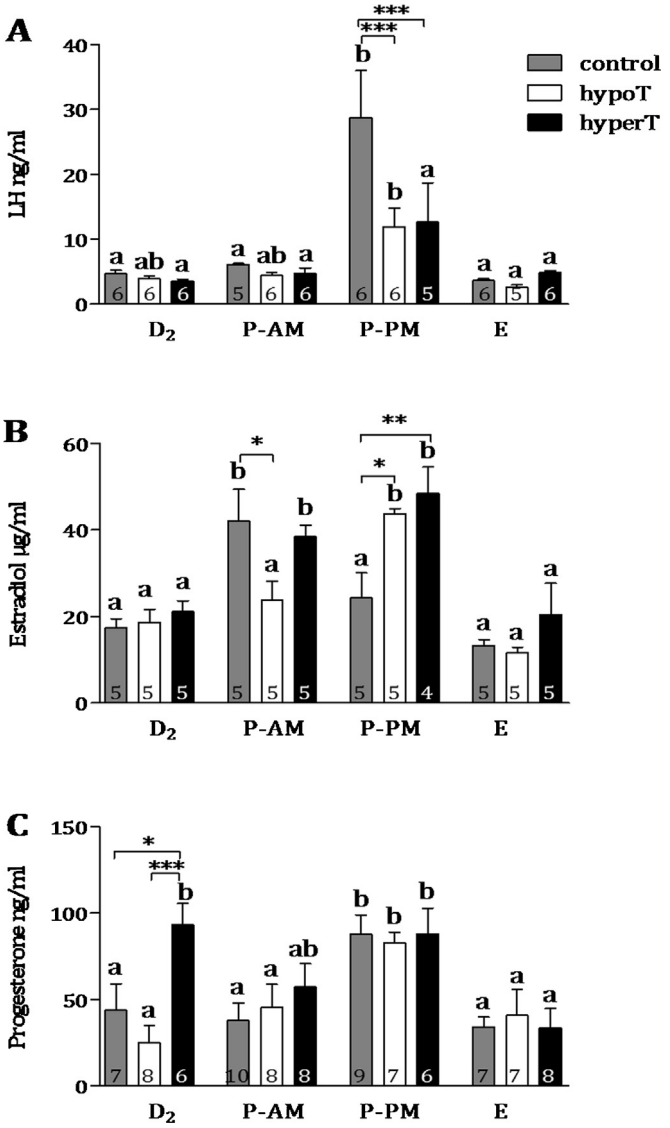

Fig. 1. Circulating luteinizing hormone (LH), estradiol and progesterone levels in control, hypoT and hyperT cycling female rats during the estrous cycle. D_2, morning of the second day of diestrus; P-AM, morning of the proestrus day; P-PM, afternoon of the proestrus day; E, morning of the estrus day; hypoT, hypothyroid rats; hyperT, hyperthyroid rats. (A) LH; (B) estradiol; (C) progesterone. Each column represents mean ± SEM. The number of samples for each group is shown at the base of each column. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ comparing the different groups within the same time point. Different superscript letters represent significant differences at $p < 0.05$ between different times/days of the cycle of the same experimental group.

Table 1
Circulating hormone levels of TSH in the groups of control and treated female rats during the estrous cycle.[a]

Group	TSH (ng/ml)			
	D_2	P-AM	P-PM	E
Control	2.35 ± 0.15	3.51 ± 0.26	3.00 ± 0.10	2.74 ± 0.18
HypoT	9.70 ± 0.40***	12.90 ± 0.41***	11.17 ± 0.34***	7.68 ± 0.62***
HyperT	2.25 ± 0.18	1.75 ± 0.04***	1.81 ± 0.06***	2.23 ± 0.07

[a] Each value represents mean ± SEM.
*Comparison of control vs. treated groups at the same time studied.
*** $p < 0.001$.

Table 2
Circulating hormone levels of T_3 and T_4 in the P-AM in control and treated groups.[a]

Group	T_3 (ng/dl)	T_4 (μg/dl)
Control-P-AM	127.70 ± 21.12	4.09 ± 0.76
HypoT-P-AM	34.19 ± 8.29***	0.85 ± 0.39**
HyperT-P-AM	273.60 ± 34.38*	75.63 ± 4.23***

[a] Each value represents mean ± SEM.
*Comparison of control vs. treated groups at the same time studied.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

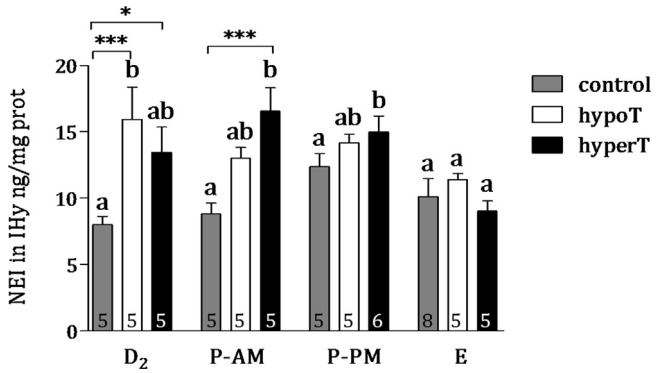

Fig. 2. NEI concentration in the incerto-hypothalamic area (IHy) during the estrous cycle. Each column represents mean ± SEM. The number of samples for each group is shown at the base of each column. For further details see legend to Fig. 1. *$p < 0.05$; ***$p < 0.001$ comparing the different groups within the same time point. Different superscript letters represent significant differences at $p < 0.05$ between different times/days of the cycle of the same experimental group.

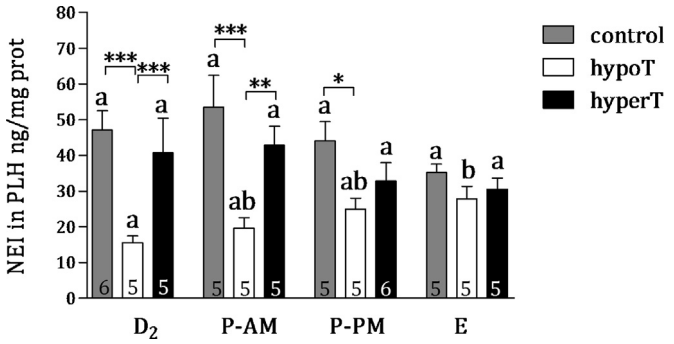

Fig. 3. NEI concentration in the peduncular part of the lateral hypothalamus (PLH) during the estrous cycle. Each column represents mean ± SEM. The number of samples for each group is shown at the base of each column. For further details see legend to Fig. 1. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ comparing the different groups within the same time point. Different superscript letters represent significant differences at $p < 0.05$ between different times/days of the cycle of the same experimental group.

In the PeFLH, control rats showed significantly elevated NEI levels on P-PM, in coincidence with the preovulatory LH peak (Fig. 4); hyperT rats, however, showed increased levels on P-AM and on E, and values significantly lower than controls on P-PM (Fig. 4). In the hypoT rats NEI concentrations were lower than controls in P-PM and significantly higher on E (Fig. 4).

3.3. NEI concentration in brain areas with NEI fibers

In the OVLT + AVPV, NEI concentration was lower in control rats at P-PM than in D_2 and P-AM. However, this difference was not observed in both groups of treated rats, that showed similar concentrations in all time points (Fig. 5). On P-PM NEI levels were higher in hypoT compared to controls (Fig. 5).

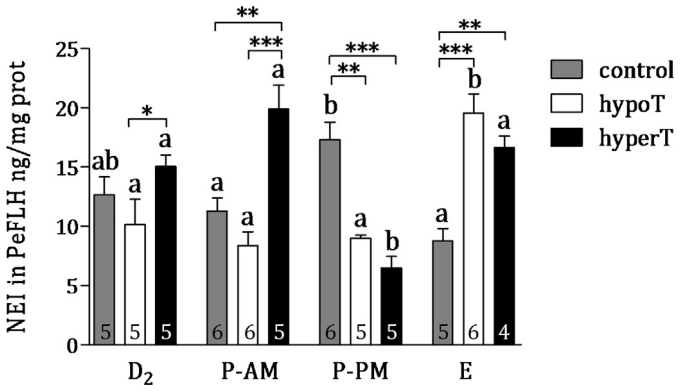

Fig. 4. NEI concentration in the perifornical part of the lateral hypothalamus (PeFLH) during the estrous cycle. Each column represents mean ± SEM. The number of samples for each group is shown at the base of each column. For further details see legend to Fig. 1. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ comparing the different groups within the same time point. Different superscript letters represent significant differences at $p < 0.05$ between different times/days of the cycle of the same experimental group.

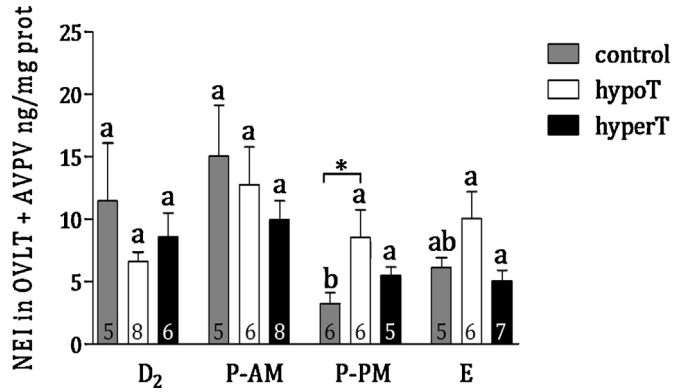

Fig. 5. NEI concentration in the organum vasculosum of the lamina terminalis and anteroventral periventricular nucleus (OVLT + AVPV) during the estrous cycle. Each column represents mean ± SEM. The number of samples for each group is shown at the base of each column. For further details see legend to Fig. 1. *$p < 0.05$ comparing the different groups within the same time point. Different superscript letters represent significant differences at $p < 0.05$ between different times/days of the cycle of the same experimental group.

In ME + Arc area, NEI concentrations showed a peak in E in control and hypoT rats. In contrast, hyperT rats showed higher NEI concentrations at P-PM and significantly lower at E compared to controls. However, no differences were observed between the different days of the cycle for hyperT groups (Fig. 6).

3.4. NEI concentration in the posterior pituitary

In the PP of control rats, NEI values were elevated in P-AM and E than at the other studied time points. In the hypoT rats the values were lower than controls in P-AM, but were similar to controls in the other time points (Fig. 7). However, in the hyperT rats the peaks observed in P-AM and E in controls were abolished and NEI concentrations were also lower than controls in P-PM (Fig. 7).

4. Discussion

In spontaneous ovulators such as rats, mice, guinea pigs, sheep, monkeys and women, reproductive endocrinology is characterized by the secretion of pituitary and ovarian hormones that follow a cyclic pattern and culminate in a preovulatory surge of LH preceded by an increase in estradiol levels [16,62]. This hormonal system is regulated by a dimorphic limbic-hypothalamic neural

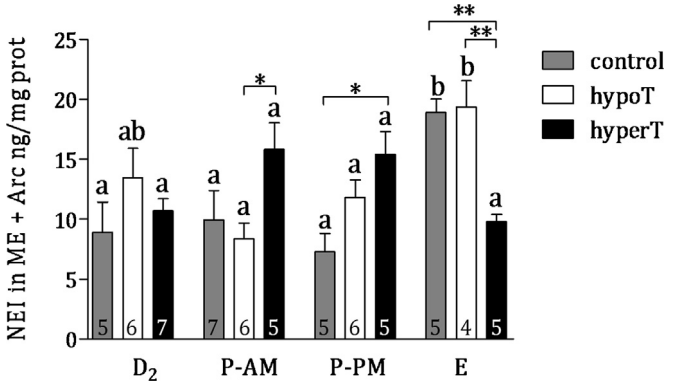

Fig. 6. NEI concentration in the median eminence and arcuate nucleus (ME + Arc) during the estrous cycle. Each column represents mean ± SEM. The number of samples for each group is shown at the base of each column. For further details see legend to Fig. 1. $^{*}p < 0.05$; $^{**}p < 0.01$ comparing the different groups within the same time point. Different superscript letters represent significant differences at $p < 0.05$ between different times/days of the cycle of the same experimental group.

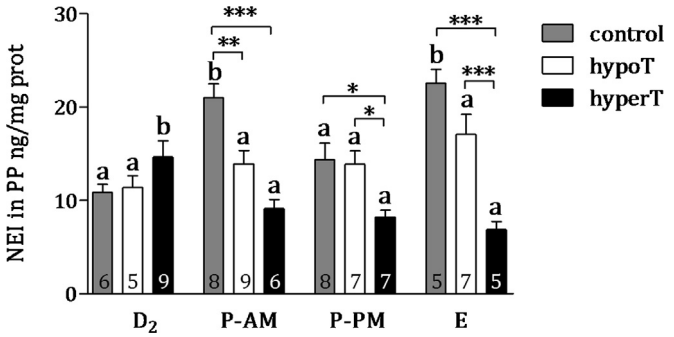

Fig. 7. NEI concentration in the posterior pituitary (PP) during the estrous cycle. Each column represents mean ± SEM. The number of samples for each group is shown at the base of each column. For further details see legend to Fig. 1. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ comparing the different groups within the same time point. Different superscript letters represent significant differences at $p < 0.05$ between different times/days of the cycle of the same experimental group.

circuit through direct and indirect actions on gonadotropin releasing hormone (GnRH) neurons [50,57]. In the present work, the serum pattern of LH, estradiol and progesterone throughout the estrous cycle of control animals showed the expected increased estradiol levels during P-AM and higher progesterone and LH values during P-PM. The pattern observed in hypoT and hyperT rats was different, evidencing a treatment effect. In hypoT rats, an increase of estradiol levels was found during P-PM, suggesting a delay of the peak observed during P-AM in the control rats. In the hyperT animals high estradiol levels persisted during P-PM, and a rise in progesterone was also observed on D_2. The reduced LH levels observed in hypoT and hyperT during P-PM may be related to the altered pattern observed in estradiol values in both treatment groups. Previous studies have shown that progesterone modulates the magnitude of the LH surge [40], for which a preceding increase of estradiol is necessary [25]. Also, estradiol regulates the expression of progesterone receptors at the AVPV and in other discrete hypothalamic areas related to female reproduction and sexual behavior [70]. Estradiol levels were modified both in hypoT and hyperT, although in a different manner. In addition, hyperT had elevated progesterone levels during D_2, which could alter the normal effect of estradiol on the induction of progesterone receptors at the hypothalamic level during the P day. The reduction in the LH surges may be a consequence of these alterations, which – as expected – may lead to ovulatory disturbances in both cases. It is important to note that the changes in gonadotropin levels during hypo- and hyperthyroidism were in agreement with previously published works [20,24,32,67].

NEI concentrations were studied in different hypothalamic areas related to its synthesis (IHy, PLH and PeFLH), and others with known presence of NEI fibers (AVPV, OVLT, ME and Arc) and the PP, where NEI fibers have also been identified.

IHy is an integrative region that is considered part of the medial hypothalamus and is possibly involved in the regulation of neuroendocrine functions, such as reproduction and sexual behavior [60]. In the IHy of euthyroid rats, peptide concentration remained unchanged throughout the estrous cycle, and there was no correlation between the studied circulating hormones and peptide concentration in this area. It is possible that the regulation exerted by gonadal steroids on NEI synthesis occurs on the projections of the IHy, such as those located at the AVPV or the ME, and not directly on the somas. The hypoT group showed higher NEI levels during D_2 than on the other days of the estrous cycle, and they were also higher than in controls on that day. It is not known whether the central thyroid hormone homeostasis mechanism shown by Kundu et al. in male rats (detection of low circulating thyroid hormones levels, increase of central conversion of T_4 to T_3 that results in significantly higher transitory T_3 levels than in controls) [34–38] exists in female rats. If so, it is probable that in female hypoT rats, normal to increased T_3 concentrations in the CNS would induce a higher synthesis of NEI and its consequent accumulation, as observed in this study in hypoT during D_2. Also, higher NEI concentrations in the IHy of hyperT rats than in controls were found during D_2 and P-AM, which may be the consequence of an increased T_3 concentration at the level of the CNS due to the external administration of L-T_4. In both cases, thyroid hormones probably affect NEI concentration by inducing an increase of NEI synthesis or its diminished degradation.

The PLH is a large and heterogeneous region of the rat hypothalamus. Physiologically it is involved in the control of several functions such as feeding, sleep–wake cycle, processing of sensitive information and modulation of motor responses associated to hunger, thirst, aggression and reproduction [8,11,14,19,23,65]. The results obtained in female rats indicate that NEI concentrations in the PLH do not change during the estrous cycle in control and hyperT rats, which showed a similar pattern. A different pattern was, however, observed in hypoT rats, which had higher values during E than during D_2; and lower levels during D_2 and P when compared to control rats. These reduced NEI concentrations show that in females, short-term hypothyroidism is able to modify the peptide content. In male rats, however, no differences were observed between control and hypoT in this area [4]. These results suggest that the regulation of NEI differs between female and male rats. Taking into account the inhibitory action of TRH on MCH (NEI) synthesizing cells [69], it is possible that in female hypoT rats, an increase of TRH induces an inhibitory action on NEI synthesis and its cleavage in the PLH.

The PeFLH is a region involved in several functions, such as feeding behavior [12,30,63], locomotion [46,59], cardiovascular regulation [64] and control of the sleep–wake cycle [1]. NEI concentrations changed in a different way for each studied group; in euthyroid rats a cyclic variation in peptide concentration was observed, with an increase during P-PM in agreement with the rise in progesterone and LH observed at this moment of the cycle. The increase in NEI concentrations in control-P-PM could be related to the increased motor activity that is characteristic at this time of the estrous cycle, as previous works had shown that NEI induces locomotor activity [53,54,56]. Also, taking into account that in female rats injection of NEI in the medial preoptic area (MPA) enhances female sexual receptivity [18], the MPA projects to the PeFLH [42], and that oxitocinergic neurons in this area are sensitive to estradiol [29], it is likely that NEI in the PeFLH may be affected by changes in gonadal steroids during the cycle and participates in the

induction of female sexual behavior. HypoT and hyperT rats showed altered cyclic patterns in PeFLH NEI content, in accordance with gonadal steroid variations that may have led to an earlier increase in NEI concentration in the hyperT (during P-AM) rats, and a delayed increase pattern (during E) in the hypoT compared with the euthyroid rats, probably affecting LH release and sexual behavior.

NEI was also studied in areas with known presence of peptide fibers. The OVLT and the AVPV are particularly involved in reproductive control, the latter playing a main role in integrating the positive feedback exerted by estradiol and progesterone to induce the LH surge [57,58]. The GnRH neurons responsible for the induction of the LH surge, and NEI fibers related to them, are located in the same regions [3,27,45,57,66,68]. In the present study, NEI concentrations in controls showed a cyclic pattern of variation, with lower peptide content during P-PM. This pattern was not observed in the hypoT and hyperT groups. It is possible that the changes in gonadal steroids observed in hypoT and hyperT rats also influence peptide action on GnRH neurons and, consequently, affect in part the LH surge induction. As an alternative, there may be a direct effect of the deficit or excess of T_3 on NEI concentration.

The ME and the Arc are two discrete areas located in the third ventricular walls. The ME is one of the circumventricular organs where specialized ependimal cells, the tanycytes [6,52], are present instead of the blood brain barrier, providing access to the portal vessels where different terminals originating in other hypothalamic areas release their factors [13,26], and isolating it from the cerebrospinal fluid and the Arc [52]. NEI axons, like many others, such as those of the GnRH, project to the ME [3,10]. The Arc is a heterogeneous region where tanycytes are also located [39,52]. It is involved in the regulation of GnRH secretion, has high expression of estradiol alpha receptor, and is implicated in the regulation of sexual behavior [48]. In the ME + Arc, higher NEI concentrations were observed during E in controls than on the other days of the cycle, which could be secondary to an increased peptide release in other regions and accumulation in ME + Arc. However, studying ME and Arc individually may provide more precise information about the implications of the concentration and action of NEI during the estrous cycle in these areas. Previous work had shown that at the ME, NEI and GnRH terminals are in close apposition between them and with portal vessels [3]. The relationship between these terminals with tanycytes may therefore provide interesting information about their interaction and the implications on the induction of the LH surge, the regulation of the estrous cycle, and the effects of THs on NEI content and terminals. Regarding the tanycytes, it has been suggested that these cells play an integral role in the regulation of the HPT axis, having one of the highest concentrations of type 2 iodothyronine deiodinase (D2) in the brain, as well as in the regulation of the HPG axis [39]. They are therefore an important target for future studies in the regulation of NEI in these areas. In the hypoT group, NEI content was similar to controls. The detection of the diminished circulating T_4 levels by tanycytes induced a rapid response through a compensatory increase of D2 by means of a conversion of T_4 to T_3, as discussed above [34–38], which may tend to normalize local T_3 levels and result in no change in NEI patterns. In contrast to this, the results observed in the hyperT group suggest that in this condition, the system may not be able to compensate altered THs levels, affecting the NEI pattern; the increased NEI levels in this group during P-PM instead of E may be one of the possible factors that alter the normal function of the Arc in the induction of the LH surge in hyperT rats. This is in agreement with the work of Freeman et al. who showed that hyperthyroidism affects the normal pattern of LH release because of an Arc dysfunction [17].

In the PP, NEI showed a cyclic pattern in controls that was modified both by hypothyroidism and hyperthyroidism. The relationship between NEI and oxytocin in this gland and the implications for reproduction should be investigated in future studies.

In this work we have shown that NEI concentration is regulated in a complex manner in different discrete brain areas. We have also shown that hypothalamic areas seem to be more sensitive to circulating hormone levels in females than in males. Furthermore, we described that NEI concentrations at different stages of the estrous cycle and its possible actions on reproduction and sexual behavior in female rats are affected by hypo- and hyperthyroidism, either indirectly through the action of gonadal steroids or directly through the deficit or excess of thyroid hormones. The impairments observed in the hypothalamic–pituitary–gonadal axis in hypoT and hyperT rats might therefore be partially mediated by NEI. It would be interesting to study the influence of NEI action impairment by hypothyroidism and hyperthyroidism at the CNS level on complex reproductive behaviors, such as sexual receptivity and locomotion.

Acknowledgements

This work was supported by grants from CONICET PIP N° 11220080102298, PICT-32529/2005 Trianual. FONCYT, Proyecto 06/J336, Universidad Nacional de Cuyo and Secretaría de Ciencia y Técnica-Universidad Nacional de Córdoba, Argentina. CA, GEP, MS, NBC, GAJ y SRV are members of CONICET. They thank Dr. Mariella Superina for revising the English language of the manuscript.

References

[1] Alam MN, Gong H, Alam T, Jaganath R, McGinty D, Szymusiak R. Sleep–waking discharge patterns of neurons recorded in the rat perifornical lateral hypothalamic area. J Physiol 2002;538(Pt 2):619–31.
[2] Attademo AM, Sanchez-Borzone M, Lasaga M, Celis ME. Intracerebroventricular injection of neuropeptide EI increases serum LH in male and female rats. Peptides 2004;25:1995–9.
[3] Attademo AM, Rondini TA, Rodrigues BC, Bittencourt JC, Celis ME, Elias CF. Neuropeptide glutamic acid-isoleucine may induce luteinizing hormone secretion via multiple pathways. Neuroendocrinology 2006;83:313–24.
[4] Ayala C, Valdez SR, Morero ML, Soaje M, Carreño NB, Sanchez MS, et al. Hypo- and hyperthyroidism affect NEI concentration in discrete brain areas of adult male rats. Peptides 2011;32(6):1249–54.
[5] Baker B, Levy A, Hall L, Lightman S. Cloning and expression of melanin-concentrating hormone genes in the rainbow trout brain. Neuroendocrinology 1995;61:67–76.
[6] Banks WA. Brain meets body: the blood-brain barrier as an endocrine interface. Endocrinology 2012;153(9):4111–9.
[7] Berberian V, Sanchez MS, Celis ME. Participation of the cholinergic system in the excessive grooming behavior induced by neuropeptide (N) glutamic acid (E) isoleucine (I) amide (NEI). Neurochem Res 2002;27:1713–7.
[8] Bernardis LL, Bellinger LL. The lateral hypothalamic area revisited: ingestive behavior. Neurosci Biobehav Rev 1996;20(2):189–287.
[9] Bittencourt JC, Elias CF. Melanin-concentrating hormone neuropeptide EI projections from the lateral hypothalamic area and zona incerta to the medial septal nucleus and spinal cord: a study using multiple neuronal tracers. Brain Res 1998;805:1–19.
[10] Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, et al. The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. J Comp Neurol 1992;319:218–45.
[11] Burdakov D, Gerasimenko O, Verkhratsky A. Physiological changes in glucose differentially modulate the excitability of hypothalamic melanin-concentrating hormone and orexin neurons in situ. J Neurosci 2005;25(9):2429–33.
[12] Burt J, Alberto CO, Parsons MP, Hirasawa M. Local network regulation of orexin neurons in the lateral hypothalamus. Am J Physiol Regul Integr Comp Physiol 2011;301(3):R572–80.
[13] Chiocchio SR, Gallardo MG, Louzan P, Gutnisky V, Tramezzani JH. Melanin-concentrating hormone stimulates the release of luteinizing hormone-releasing hormone and gonadotropins in the female rat acting at both median eminence and pituitary levels. Biol Reprod 2001;64(5):1466–72.
[14] Conductier G, Nahon J, Guyon A. Dopamine depresses melanin concentrating hormone neuronal activity through multiple effects on α2-noradrenergic, D1 and D2-like dopaminergic receptors. Neuroscience 2011;178:89–100.
[15] De Paul AL, Attademo AM, Carón RW, Soaje M, Torres AI, Jahn GA, et al. Neuropeptide glutamic-isoleucine (NEI) specifically stimulates the secretory activity of gonadotrophs in primary cultures of female rat pituitary cells. Peptides 2009;30:2081–7.

[16] Freeman M. The neuroendocrine control of the ovarian cycle of the rat. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York: Raven Press; 1994. p. 613–58.
[17] Freeman ME, LaRochelle Jr FT, Moore RB. Effect of thyroid status on spontaneous and induced surges of luteinizing hormone. Endocrinology 1976;99(3):713–9.
[18] Gonzalez MI, Baker BI, Hole DR, Wilson CA. Behavioral effects of neuropeptide E-I (NEI) in the female rat: interactions with alpha-MSH, MCH and dopamine. Peptides 1998;19:1007–16.
[19] Guyon A, Conductier G, Rovere C, Enfissi A, Nahon JL. Melanin-concentrating hormone producing neurons: activities and modulations. Peptides 2009;30(11):2031–9.
[20] Hapon MB, Gamarra-Luques C, Jahn GA. Short term hypothyroidism affects ovarian function in the cycling rat. Reprod Biol Endocrinol 2010;11:4–8.
[21] Hapon MB, Simoncini M, Via G, Jahn GA. Effect of hypothyroidism on hormone profiles in virgin, pregnant and lactating rats, and on lactation. Reproduction 2003;126:371–82.
[22] Hapon MB, Varas SM, Jahn GA, Gimenez MS. Effects of hypothyroidism on mammary and liver lipid metabolism in virgin and late-pregnant rats. J Lipid Res 2005;46:1320–30.
[23] Hassani OK, Lee MG, Jones BE. Melanin-concentrating hormone neurons discharge in a reciprocal manner to orexin neurons across the sleep–wake cycle. Proc Natl Acad Sci USA 2009;106(7):2418–22.
[24] Hatsuta M, Abe K, Tamura K, Ryuno T, Watanabe G, Taya K, et al. Effects of hypothyroidism on the estrous cycle and reproductive hormones in mature female rat. Eur J Pharmacol 2004;486(3):343–8.
[25] Herbison AE. Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V). Brain Res Rev 2008;57(2):277–87.
[26] Herde MK, Geist K, Campbell RE, Herbison AE. Gonadotropin-releasing hormone neurons extend complex highly branched dendritic trees outside the blood–brain barrier. Endocrinology 2011;152(10):3832–41.
[27] Ibanez MA, Gu G, Simerly RB. Target-dependent sexual differentiation of a limbic-hypothalamic neural pathway. J Neurosci 2001;21(15):5652–9.
[28] Jahn GA, Moya G, Jammes H, Rosato RR. Effect of chronic thyroid hormone treatment on cycling, ovulation, serum reproductive hormones and ovarian LH and prolactin receptors in rats. Endocrine 1995;3(2):121–7.
[29] Jirikowski GF, Caldwell JD, Pedersen CA, Stumpf WE. Estradiol influences oxytocin-immunoreactive brain systems. Neuroscience 1988;25(1):237–48.
[30] Jo YH, Wiedl D, Role LW. Cholinergic modulation of appetite-related synapses in mouse lateral hypothalamic slice. J Neurosci 2005;25(48):11133–44.
[31] Kennedy AR, Todd JF, Stanley SA, Abbott CR, Small CJ, Ghatei MA, et al. Melanin-concentrating hormone (MCH) supresses thyroid stimulating hormone (TSH) release, in vivo and in vitro, via the hypothalamus and the pituitary. Endocrinology 2000;142:3265–8.
[32] Krassas GE, Poppe K, Glinoer D. Thyroid function human reproductive health. Endocr Rev 2010;31:1–54.
[33] Kruger NJ. The Bradford method for protein quantitation. Methods Mol Biol 1994;32:9–15.
[34] Kundu S, Biswas A, Roy S, De J, Pramanik M, Ray AK. Thyroid hormone homeostasis in brain: possible involvement of adrenergic phenomenon in adult rat. Neuroendocrinology 2009;89:140–51.
[35] Kundu S, Pramanik M, Roy S, De J, Biswas A, Ray AK. Maintenance of brain thyroid hormone level during peripheral hypothyroid condition in adult rat. Life Sci 2006;79:1450–5.
[36] Kundu S, Ray AK. Thyroid hormone homeostasis in adult mammalian brain: a novel mechanism for functional preservation of cerebral T_3 content during initial peripheral hypothyroidism. Al Ameen J Med Sci 2010;3(1):5–20.
[37] Kundu S, Roy S, Biswas A, De J, Pramanik M, Ray AK. Possible influence of dopaminergic receptor in maintenance of thyroid hormone homeostasis: a study in adult rat brain. J Endocrinol Metab 2012;2(3):128–34.
[38] Kundu S, Roy S, De J, Biswas A, Pramanik M, Ray AK. Maintenance of homeostasis for thyroid hormone in the adult rat brain: possible involvement of a nuclear-mediated phenomenon. Neuroendocrinology 2007;86(2):94–103.
[39] Lechan RM, Fekete C. Infundibular tanycytes as modulators of neuroendocrine function: hypothetical role in the regulation of the thyroid and gonadal axis. Acta Biomed 2007;78(Suppl 1):84–98.
[40] Lee WS, Smith MS, Hoffman GE. Luteinizing hormone-releasing hormone neurons express Fos protein during the proestrous surge of luteinizing hormone. Proc Natl Acad Sci USA 1990;87:5163–7.
[41] Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol 2002;62(4A):609–14.
[42] Marson L, Murphy AZ. Identification of neural circuits involved in female genital responses in the rat: a dual virus and anterograde tracing study. Am J Physiol Regul Integr Comp Physiol 2006;291(2):R419–28.
[43] Mattheij JA, Swarts JJ, Lokerse P, van Kampen JT, Van der Heide D. Effect of hypothyroidism on the pituitary–gonadal axis in the adult female rat. J Endocrinol 1995;146(1):87–94.
[44] Nahon JL, Presse F, Bittencourt JC, Sawchenko PE, Vale W. The rat melanin-concentrating hormone messenger ribonucleic acid encodes multiple putative neuropeptides coexpressed in the dorsolateral hypothalamus. Endocrinology 1989;125:2056–65.
[45] Orikasa C, Kondo Y, Hayashi S, McEwen BS, Sakuma Y. Sexually dimorphic expression of estrogen receptor beta in the anteroventral periventricular nucleus of the rat preoptic area: implication in luteinizing hormone surge. Proc Natl Acad Sci USA 2002;99(5):3306–11.
[46] Parker SM, Sinnamon HM. Forward locomotion elicited by electrical stimulation in the diencephalon and mesencephalon of the awake rat. Physiol Behav 1983;31(5):581–7.
[47] Parkes D, Vale W. Secretion of melanin-concentrating hormone and neuropeptide-EI from cultured rat hypothalamic cells. Endocrinology 1992;131:1826–31.
[48] Patisaul HB, Fortino AE, Polston EK. Sex differences in serotonergic but not gamma-aminobutyric acidergic (GABA) projections to the rat ventromedial nucleus of the hypothalamus. Endocrinology 2008;149(1):397–408.
[49] Paxinos G, Atlas WC. The rat brain in stereotaxic coordinates. 7th ed. London: Elsevier Inc; 2007.
[50] Polston EK, Simerly RB. Ontogeny of the projections from the anteroventral periventricular nucleus of the hypothalamus in the female rat. J Comp Neurol 2006;495(1):122–32.
[51] Presse F, Nahon JL, Fischer WH, Vale W. Structure of the human melanin concentrating hormone mRNA. Mol Endocrinol 1990;4:632–7.
[52] Rodríguez EM, Blázquez JL, Guerra M. The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: the former opens to the portal blood and the latter to the cerebrospinal fluid. Peptides 2010;31(4):757–76.
[53] Sanchez M, Baker BI, Celis ME. Melanin-concentrating hormone (MCH) antagonizes the effects of alpha-MSH and neuropeptide E-I on grooming and locomotor activities in the rat. Peptides 1997;18:393–6.
[54] Sanchez MS, Barontini M, Armando I, Celis ME. Correlation of increased grooming behavior and motor activity with alterations in nigrostriatal and mesolimbic catecholamines after alpha-melanotropin and neuropeptide glutamine-isoleucine injection in the rat ventral tegmental area. Cell Mol Neurobiol 2001;21:523–33.
[55] Sanchez MS, Celis ME, Schioth HB. Evidence that alpha-MSH induced grooming is not primarily mediated by any of the cloned melanocortin receptors. Neuropeptides 2000;34:77–82.
[56] Sánchez-Borzone ME, Attademo A, Baiardi G, Celis ME. Effect of beta-adrenoceptors on the behaviour induced by the neuropeptide glutamic acid isoleucine amide. Eur J Pharmacol 2007;568:186–91.
[57] Simerly RB. Organization and regulation of sexually dimorphic neuroendocrine pathways. Behav Brain Res 1998:92195–203.
[58] Simerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol 1990;294(1):76–95.
[59] Sinnamon HM, Karvosky ME, Ilch CP. Locomotion and head scanning initiated by hypothalamic stimulation are inversely related. Ann N Y Acad Sci 1998;860:560–2.
[60] Sita L, Elias C, Bittencourt J. Connectivity pattern suggests that incerto-hypothalamic area belongs to the medial hypothalamic system. Neuroscience 2007;148(4):949–69.
[61] Skofitsch G, Jacobowitz DM, Zamir N. Immunohistochemical localization of a melanin concentrating hormone-like peptide in the rat brain. Brain Res Bull 1985;15:635–49.
[62] Smith MS, Freeman ME, Neill JD. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 1975;96(1):219–26.
[63] Stanley BG, Magdalin W, Seirafi A, Thomas WJ, Leibowitz SF. The perifornical area: the major focus of (a) patchily distributed hypothalamic neuropeptide Y-sensitive feeding system(s). Brain Res 1993;604(1-2):304–17.
[64] Stock G, Rupprecht U, Stumpf H, Schlör KH. Cardiovascular changes during arousal elicited by stimulation of amygdala, hypothalamus and locus coeruleus. J Auton Nerv Syst 1981;3(2-4):503–10.
[65] Swanson LW. The hypothalamus. In: Bjorklund A, Hokfelt T, Swanson LW, editors. Handbook of chemical neuroanatomy, 5. Amsterdam: Elsevier; 1987. p. 1–124.
[66] Szawka RE, Poletini MO, Leite CM, Bernuci MP, Kalil B, Mendonça LB, et al. Release of norepinephrine in the preoptic area activates anteroventral periventricular nucleus neurons and stimulates the surge of luteinizing hormone. Endocrinology 2013;154(1):363–74.
[67] Tohei A. Studies on the functional relationship between thyroid, adrenal and gonadal hormones. J Reprod Dev 2004;50:9–20.
[68] Watson Jr RE, Langub Jr MC, Engle MG, Maley BE. Estrogen-receptive neurons in the anteroventral periventricular nucleus are synaptic targets of the suprachiasmatic nucleus and peri-suprachiasmatic region. Brain Res 1995;689(2):254–64.
[69] Zhang X, van den Pol AN. Thyrotropin-releasing hormone (TRH) inhibits melanin-concentrating hormone neurons: implications for TRH-mediated anorexic and arousal actions. J Neurosci 2012;32(9):3032–43.
[70] Zuloaga DG, Yahn SL, Pang Y, Quihuis AM, Oyola MG, Reyna A, et al. Distribution and estrogen regulation of membrane progesterone receptor-β in the female rat brain. Endocrinology 2012;153(9):4432–43.