EFFECTS OF ESTROUS CYCLE AND SEX ON THE EXPRESSION OF NEUROPEPTIDE Y Y1 RECEPTOR IN DISCRETE HYPOTHALAMIC AND LIMBIC NUCLEI OF TRANSGENIC MICE

EFFECTS OF ESTROUS CYCLE AND SEX ON THE EXPRESSION OF NEUROPEPTIDE Y Y1 RECEPTOR IN DISCRETE HYPOTHALAMIC AND LIMBIC NUCLEI OF TRANSGENIC MICE

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Running title: Estrous cycles and Y1 expression

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Key words: medial preoptic nucleus, ventromedial nucleus, arcuate nucleus, bed nucleus of stria terminalis, gonadal hormones, sex dimorphism.

ABSTRACT

In the present study we used a transgenic mouse model, carrying the neuropeptide Y (NPY) Y1 receptor gene promoter linked to the *LacZ* reporter gene (Y1R/*LacZ* mice) to test the hypothesis of its up-regulation by gonadal hormones. Y1 receptor gene expression was detected by means of histochemical procedures and quantitative image analysis in the paraventricular nucleus, arcuate nucleus, medial preoptic nucleus, ventromedial nucleus and bed nucleus of stria terminalis of two month-old female mice at different stages of estrous cycle. Qualitative and quantitative analises showed that Y1R/*LacZ* transgene expression was higher in the paraventricular, arcuate, and ventromedial nuclei of proestrus mice as compared to mice in the other stages of the estrous cycle. In addition, we performed a comparison with a group of sexually active males. In this comparison a significant difference (less in males) was observed between males and proestrus females in the same nuclei. In conclusion, these data indicate that fluctuations in circulating levels of gonadal hormones, depending by estrous cycle, are paralleled by changes in the expression of NPY Y1 receptor in the hypothalamic nuclei involved in the control of both energy balance and reproduction.

1. INTRODUCTION

Neuropeptide Y (NPY) is a 36 aminoacid peptide widely expressed throughout the central nervous systems (CNS) of vertebrates. In particular, NPY is present in key centers for the regulation of endocrine and neuroendocrine functions (for a review see [8]) as in the hypothalamic arcuate nucleus (ARC, [22]), and in the amygdala [12]). NPY neurons of the ARC mostly project to the paraventricular nucleus (PVN, the main site of the control of energy balance [2]), while the NPY neurons of the amygdala belong to circuits related to stress and emotionality [34].

Due to its wide distribution, NPY is involved in a variety of biological effects grouped in four main fields: regulation of food intake [5], neuroendocrine control of reproduction and of sexual behavior [1], depression [29], anxiety and stress [33].

The intracerebroventricular injection of NPY caused a dose-related inhibition of copulatory behavior in adult sexually-experienced male rats [28], and inhibition of lordosis in ovariectomized steroid-primed female Syrian hamsters [7]. On the other hand, the administration of a NPY antagonist improves sexual behavior in male rats [27], and attenuates the termination of receptivity in female rats [6]. Gonadal hormones can influence NPY mRNA expression, in fact, castrated male rats show a decrease of NPY concentration in ARC [36]. In turn, NPY positive neurons make synaptic contacts on GnRH neurons and fibers [35], and modulate GnRH and LH secretion [32], for reviews see [9, 38]. Finally, NPY mRNA expressing neurons are in larger number in males than in proestrus female in the caudal ARC [36] suggesting the existence of a functional sexual dimorphism.

NPY stimulates LH secretion [16] and GnRH release in rats via the activation of the Y1 receptor subtype [39]. These activities are estrogen-dependent, and they seem to be related to interactions of estrogens with the Y1-R gene [39]. In vitro studies performed on transiently transfected NG108-15 neuroblastoma-glioma cells have shown that activation of estrogen receptor alpha (ERα) stimulate transcriptional activity of the Y1-R, possibly by interacting with estrogen-responsive elements (ERE) [23]. Moreover studies in vivo demonstrated, by competitive RT-PCR, that the content of hypothalamic Y1R mRNA changes during the estrous cycle in female rats [39].

In the present study we used a transgenic mouse model, carrying the Y1 receptor (Y1-R) gene promoter linked to the *LacZ* reporter gene (*Y1R/LacZ* mice) [26], to identify the hypothalamic and limbic nuclei where changes in Y1-R take place during estrous cycle. In addition we investigated the putative existence of sexual differences in these regions.

2. MATERIALS AND METHODS

2.1 Animals

A total of 24 Y1R/*Lac Z* transgenic mice (4 males and 20 females, line 62 from our breeding colony [26]) were employed in this study. The animals were housed into monosexual groups of five per cage with food and water *ad libitum* and were maintained on a 12h light-12 h dark cycle at a temperature of 22-28°C.

The females were divided into 4 groups [proestrus (n=5), estrus (n=5), metaestrus (n=5), diestrus (n=5)], depending on the day of the cycle detected by examination of vaginal smears immediately before the sacrifice.

Animal care was in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC), and the experimental protocol was approved by animal investigation committee of Italian MIUR and by the ethic committee of the University of Torino.

2.2 Fixation and tissue preparation

At the age of two months the mice were irreversibly anaesthetized by an intraperitoneal injection of tri-bromo-ethanol (250 mg/kg) followed by trans-cardiac perfusion of a saline solution (0.9%), until the return blood was clear, and then with 150 ml of fixative [4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3-7.4].

Brains were dissected out of the skull, post-fixed for 2 h at 4°C in the same fixative, rinsed in 0.01 M saline PB (PBS), placed overnight in a 30% sucrose solution in PBS, frozen in liquid isopentane at -35°C, and stored in a deep freezer at -80° C. They were serially sectioned in the coronal planes at 25µm thickness with a cryostat. The plane of sectioning was oriented to match the drawings corresponding to the transverse sections of the mouse brain atlas [10]. Sections were collected in a cryoprotectant solution [37] at -20°C. Every fourth section (a section every 100 µm) one was

processed for Y1R histochemistry. Brain sections were always stained in groups containing male and females from all estrous cycle stages, so that between assays variance could not cause systematic group differences.

2.3 Beta-galactosidase histochemistry

Y1R/*LacZ* expression was determined by β-galactosidase staining of brain coronal sections according to our previously described method [24-26] slightly modified (due to the perfusion of the animals, the sections were not further fixed). Briefly, sections were incubated overnight at 37°C in a solution containing 1mg/ml X-gal, 5mM potassium ferricyanide, 5mMpotassium ferrocyanide, 2mMMgCl2, 0,01% Triton X-100 in 1X PBS. They were then washed in water for 5 minutes, counterstained with nuclear fast red, coverslipped with DPX mounting medium (Fluka Chemical Co., Buchs, Switzerland) and analyzed.

2.4 Quantification of transgene expression as determined by β-galactosidase histochemistry

The expression of the transgene appears as blue dots. Sections were counterstained with neutral fast red and hypothalamic nuclei were identified on the basis of the mouse brain atlas [10]. For each mouse, two standardized sections of comparable levels of the medial preoptic nucleus (MPOM) (around bregma 0.14, 0.10 mm), ventromedial nucleus (VMH) (around bregma –1.46 –1.70), ARC (around bregma –1.46 –1.70), bed nucleus of stria terminalis, pars medialis ventral portion (BSTMV) (around bregma -0.02, -0.10), and three sections of the PVN (around bregma -0.58, -0.82, -0.94) were chosen. Quantification of the *Y_1R/LacZ* transgene expression was made by computer assisted morphometrical analysis as previously described [24, 40]. Briefly, selected sections were observed by means of a x10 objective, and digitized. Image analysis was performed using the software NIH-Image (version 1.62, a freeware by W. Rasband, NIH, Bethesda, USA). Sections were at first digitized by using a built-in green filter to better identify the nuclei extension. A line, drawn following the boundaries of the selected nuclei, defined the area of interest (AOI). The same section was then digitized using a built-in red filter obtaining a strong enhancement of the histochemical signal. The AOI selected on the first image was finally superimposed on the second image to delimit the region in which dots should

be counted by using a manual thresholding method. For each animal and nucleus the cumulative number of dots and the cumulative areas of the analyzed sections were considered to obtain the average density expression of the transgene expressed as dots per μm^2. This method provides semiquantitative analysis of changes in β-galactosidase expression, reflecting changes in promoter activity.

2.5 Statistical analysis

To detect global changes in the expression of the transgene, a one-way ANOVA (being the cycle phase the independent variable) for repeated measures (the different nuclei) of the average density values of each of the four female groups plus the male group was performed. To detect differences during the female cycle and possible sexual differences, the one-way ANOVA was repeated per each nucleus including 4 female groups and male. Differences were considered statistically significant for values of $p<0,05$. These analyses were followed, when appropriate, by Fisher PLSD test. The software used was Statview 5.0 (Abacus Concepts, Berkely, CA, USA).

3. RESULTS

3.1 Distribution pattern

The distribution pattern of *Y_1R/LacZ* transgene expression in hypothalamic and limbic nuclei of male and female mice was in agreement to our previous studies. A relative large number of positive cells was observed in the PVN, ARC, VMH, and MPOA nuclei, as well as in the medial ventral portion of BST (BSTMV) [24, 26, 40]. Images typical of those subjected to computer-assisted quantitation of β-galactosidase expression are shown in Fig. 1 (top)

3.2 Quantitative analysis

The one-way ANOVA for repeated measures (being the different groups the independent variable and the different nuclei the repeated measures) reported significant effects for all considered parameters: group effect [$F_{(4,20)}= 4.40$, $p=0.01$], nucleus effect [$F_{(4,4,20)}=56.4$, $p<0.0001$] and also interaction effect [$F_{(4,4,16)}=2.17$, $p=0.015$]. Taking into consideration the densities' values of the different nuclei together, the post-hoc Fisher PLSD test reported significant differences among males

and proestrus females ($p=0.012$), and proestrus females against estrus ($p<0.05$), metaestrus and diestrus ones ($p<0.002$) (Supplementary Fig. 1).

The one-way ANOVA for each nucleus (Fig.1, bottom) confirmed that in all considered nuclei, the highest density of the Y1R/LacZ transgene expression was detected in proestrus females as compared to estrus, metaestrus and diestrus ones, and to males. A significant effect of cycle was detected in PVN [$F_{(4,20)}=3.65$, $p=0.02$], VMH [$F_{(4,20)}=4.99$, $p=0.006$], and ARC [$F_{(4,20)}=11.10$, $p<0.0001$]. A tendency to significant difference was observed in the BSTMV [$F_{(4,20)}=2.621$, $p=0.065$] whereas no significant differences among groups were measured in the MPOM [$F_{(4,20)}=0.69$ $p=0.60$].

When compared to the other phases of the estrous cycle the expression of the transgene in proestrus was significantly higher in VMH and ARC. In the PVN a significant difference was detected only for comparisons between proestrus versus metestrus and versus diestrus. No differences in transgene expression were detected for the comparison proestrus-estrus. We examined the post-hoc test's results also for BSTMV, and in this case a significant difference was detected for comparison proestrus versus metestrus and diestrus, in addition the transgene density in estrus was significantly higher than diestrus. The transgene expression in male group was significantly lower than proestrus female in PVN ($p=0.016$), ARC ($p=0.0002$), and VMH ($p=0.011$). No other comparison was significant.

4. **DISCUSSION**

The central event of the female reproductive cycle, ovulation, depends on the coordinated release of pituitary gonadotropin and modulatory factors. Several studies demonstrated that NPY is critically important in the neuronal regulation of the GnRH secretions and that Y1R is implicated in the augmentation of LH release (for a review see [14]). Demonstration that NPY acts thought the Y1R subtype to stimulate LH preovulatory surge was first drawn from the observation that specific pharmacological activation of Y1R stimulates the LH surge in proestrus rats [19]. NPY-induced augmentation of GnRH release during proestrus involves a dramatic increase of tissue responsiveness to NPY. Finally, this effect requires a proestrus hormonal environment [3]. Levine and coauthors [13, 39] have shown that the influence of the steroid environment may affect Y1R-mediated signalling and that estrogens up-regulate

responsiveness to NPY through regulation of Y1R gene expression in the hypothalamus. This was demonstrated by the observation that Y1R mRNA expression is significantly increased in the hypothalamus of proestrus rats and that a similar up-regulation can be induced by exogenous estrogen treatment.[18, 39].

With the use of Y1R/LacZ transgenic mice, we have here shown that Y1R gene expression is significantly higher in hypothalamus of proestrus mice as compared to mice in all the other phases of the estrous cycle. Specifically, the up-regulation of the Y1R gene expression was observed in PVN, VMH, ARC whereas low or no significant differences were observed in BSTv and MPOM, respectively.

Our results showed also the presence of a sex difference of Y1R gene expression transgene in PVN, ARC and VMH nuclei, by comparison of male mice with female in proestrus, females having higher expression of the transgene. In the rat, NPY gene expression throughout the ARC is modulated by T in male rats, and a marked regional sex difference (higher in males vs proestrus females) exists in the distribution of NPY mRNA-containing cells in the caudal extremity of the ARC [36], suggesting the presence of both organizational and activational effects of gonadal hormones upon NPY expression. Our results suggest that this could be true also for Y1R expression in PVN, ARC and VMH, but in this case it is probably mediated by E_2.

At the molecular level, E_2 may increase Y1R gene expression by direct interaction of ERα with three hemipalindromic estrogen-responsive elements flaking the Y1R gene promoter, as previously suggested [23]. In accordance with our present results, other studies showed the regulation of the hypothalamic NPY-Y1 receptor mRNA during estrous cycle. Ovariectomy eliminated the increase of Y1R mRNA detected in hypothalamic extracts during the late morning or early afternoon of proestrus. E_2 treatment restores it. Moreover an involvement of progesterone receptor in stimulating Y1 receptor expression has been also demonstrated. Additional progesterone following E_2 treatment produces even larger increases in Y1 receptor mRNA levels [39]. Accordingly, we found an increase in Y1R gene expression transgene in the PVN and ARC nuclei of 18 days pregnant mice as compared with estrus mice [24]

It is interesting to notice that the hormonal changes related to estrous cycle failed to affect Y1R gene expression in those limbic nuclei (MPOM and BST) known to be important in regulating GnRH neurosecretion, which also express alfa and beta

estrogen receptors both in rats and mice [21, 30] and show estrus-dependent variations in other gonadal hormones dependent neural circuits (i.e. the nitrergic system [11, 31]).

4.1 - Conclusion

Growing evidence suggests that NPY may at least in part mediate communication among energy balance, GnRH secretion and sexual behavior. NPY consistently suppresses LH release when administered on a chronic basis, leading to the cessation of reproduction [4]. NPY synthesis and release are greatly increased in response to metabolic challenge inhibiting the pulsatile mode of LH secretion [15, 20], such as starvation and increased energy expenditure. In turn, NPY levels are reduced by treatments that ameliorate metabolic deficit and reinstate HPG function [17]. Thus regulation of Y1R gene expression induced by gonadal hormones during estrous cycle seems to be restricted to those hypothalamic nuclei that are involved in regulation of both energy balance and reproduction.

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Legend to figure

Fig.1 Top. Variations of transgene expression during the estral cycle and in the male Y1/LacZ transgenic mice. PVN paraventricular nucleus, VMH ventromedial nucleus, ARC arcuate nucleus, ME median eminence. Bar = 200 μm.

Bottom. Bar graphs illustrating changes in the density of Y1/LacZ transgene in different nuclei expressed as number of dots/$\mu m^2 x 10^3$. The groups are the different phases of the female estrous cycle and the male group. * $p<0.05$ in comparison to Proestrus **$p<0.01$ in comparison to Proestrus ***$p<0.001$ in comparison to Proestrus $^{@}$ $p<0.05$ in comparison to Estrus

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Figure 1

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