Psicothema

Psicothema 2013, Vol. 25, No. 4, 461-467
doi: 10.7334/psicothema2013.100

ISSN 0214 - 9915 CODEN PSOTEG

www.psicothema.com

Sexual metabolic differences in the rat limbic brain

Natalia Arias, Javier Morán, Nélida Conejo and Jorge L. Arias
Universidad de Oviedo

Abstract

Background: There is actually limited evidence about the influence of estrogens on neuronal energy metabolism or functional cerebral asymmetry. In order to evaluate this relationship, eight male and sixteen female adult Wistar rats, divided into estrus and diestrus phase, were used to measure basal neuronal metabolic activity in some of the structures involved in the Papez circuit, using cytochrome c oxidase (C.O.) histochemistry. **Method:** We used C.O. histochemistry because cytochrome oxidase activity can be considered as a reliable endogenous marker of neuronal activity. **Results:** We found higher C.O. activity levels in diestrus as compared to estrus and male groups in the prefrontal cortex and thalamus. Conversely, neuronal oxidative metabolism was significantly higher in estrus than in diestrus and male groups in the dorsal and ventral hippocampus (CA1 and CA3) and in the mammillary bodies. However, no hemispheric functional lateralization was found in estrus, diestrus or male groups by C.O. activity. **Conclusions:** These results suggest a modulatory effect of estrogens on neuronal oxidative metabolism.

Keywords: Estrogen, oxidative metabolism, functional cerebral asymmetry, Papez circuit, rat.

Resumen

Diferencias metabólicas sexuales en el cerebro límbico de rata. **Antecedentes:** existe poca evidencia acerca de la influencia de los estrógenos sobre el metabolismo energético cerebral o la asimetría cerebral funcional. Para evaluarlo, se utilizaron ocho machos y dieciséis hembras de rata adultas de la cepa Wistar, divididas en fase estro y diestro, con el fin de medir la actividad metabólica neuronal basal en algunas de las estructuras cerebrales del circuito de Papez. **Método:** utilizamos la histoquímica de la citocromo c oxidasa (C.O.) porque su actividad puede ser considerada como un relevante marcador endógeno de la actividad neuronal. **Resultados:** encontramos mayores niveles de C.O. en el diestro en comparación con el estro y los machos en la corteza prefrontal y el tálamo. El metabolismo oxidativo neuronal fue significativamente mayor en el estro en comparación con el grupo diestro y los machos en el hipocampo dorsal y ventral (CA1 y CA3), así como en los cuerpos mamilares. No se encontró ninguna lateralización hemisférica funcional en los grupos experimentales. **Conclusiones:** estos resultados sugieren un efecto modulador de los estrógenos sobre el metabolismo oxidativo neuronal.

Palabras clave: estrógenos, metabolismo oxidativo, asimetría funcional cerebral, circuito de Papez, rata.

Functional cerebral asymmetries (FCAs) are affected by the organizing and activating effects of sex hormones (Wisniewski, 1998). It has been shown that FCAs fluctuate over the menstrual cycle, presumably due to cycle-related hormonal variations (Sanders & Wenmoth, 1998). These FCAs could explain the different anatomical adaptations to chronic stress between female and male rats (Conrad, Grote, Hobbs, & Ferayorni, 2003), whereby females may be less vulnerable to hippocampal-dependent memory deficits after chronic stress or hippocampal damage. On the other hand, it has been reported that gonadal steroids influence learning and extinction of the conditioned reflex of passive and active avoidance (Sashkov, Sel'verova, Morenkov, & Ermakova, 2010; Rubio, Miranda, Cuesta, Begega, Santín, & Arias, 1999). For example, in relation to spatial tasks, male rats outperformed females in the water maze task where the environment contained mostly distal cues (Chai & Jacobs, 2010), whereas females outperformed males in detecting local objects whose locations had been switched (Saucier, Shurtz, Keller, Cook, & Binsted, 2008).

One approach to understand the sexual differentiation of brain and behavior arise from studies of estrogens receptors (ERs) suggesting that estrogens could directly affect the hippocampus where there are α (Koike, Sakai, & Muramatsu, 1987) and β (Kuiper, Enmark, Pelto-Huikkom, Nilsson, & Gustafsson, 1996) ERs, and the prefrontal cortex that contains ERs β (Kritzer, 2002), as both brain regions are involved in learning and memory (Conejo, González-Pardo, Vallejo, & Arias, 2007). Brain asymmetry and functional lateralization have also been observed. In laboratory rats, functional lateralization of C.O. activity by sex and estrous cycle stage in the cingulate cortex and mediodorsal thalamus were found (Arias, Álvarez, Conejo, González-Pardo, & Arias, 2010). Even at neurochemical level, for example, the asymmetric distribution of GABA binding sites in the cerebral cortex, hippocampus, cerebellar hemispheres, striatum and thalamus (Oke, Lewis, & Adams, 1980), the hippocampal nitric oxide system with its right/left lateralization (Kristofiková et al., 2008), the dopaminergic enrichment of the right brain (Afonso, Santana, & Rodríguez, 1993) and the higher abundance of metabolic enzymes related to cellular energy metabolism in the right hippocampus than the left one (Samara et al., 2011) could be good examples. Nevertheless,

Received: April 11, 2013 • Accepted: June 27, 2013
Corresponding author: Jorge L. Arias
Facultad de Psicología
Universidad de Oviedo
33003 Oviedo (Spain)
e-mail: jarias@uniovi.es

the existence of brain lateralization in rodents is still under debate because very few studies have been performed on this subject. Furthermore, there is little information about the relationship between estrogen levels and FCA, especially in non-cortical brain regions.

Therefore, the aim of this study was to evaluate functional cerebral asymmetries in female rats at two levels of the estrous cycle and in males. For this purpose, we studied oxidative metabolism of different brain limbic system regions included in the Papez circuit by histochemical labelling of cytochrome oxidase (C.O.). Estrogens play a role in regulating oxygen consumption as an index of mitochondrial respiratory complexes (MRC) (Klinge, 2008). The increase of estrogens and ERs could be involved in the enhanced expression of mitochondrial DNA-encoded MRC proteins (Chen, Yager, & Russo, 2005). One of these MRC proteins is MRC-IV (cytochrome c oxidase), which is a mitochondrial enzyme involved in the phosphorylation process that generates energy stored as ATP. As metabolic activity is tightly coupled to neuronal activity, this technique can be used as an index of regional functional activity in the brain, reflecting changes in tissue metabolic capacity induced by sustained energy requirements of the nervous system associated with the influence of sex differences in behaviour (Conejo, González-Pardo, Vallejo, & Arias, 2004).

Method

Subjects

Adult Wistar rats weighing between 225-275g were obtained from the University of Oviedo (Spain) central vivarium. They were housed in groups of three to six in standard plastic cages (27×27×15 cm) and kept at constant room temperature (23 ± 2ºC), with a relative humidity of 65 ± 5% and artificial light-dark cycle of 12h (lights on at 8:00 a.m.). Food and water were available ad libitum. The procedures and manipulation of the animals used in this study were carried out according to the Directive (2010/63/EU) and Royal Decree 1201/2005 of the Ministry of Presidency relating to the protection of the animals used for experimentation and other scientific purposes, and the study was approved by the local committee for animal studies (Oviedo University).

Procedure

After handling the animals during ten minutes, they were tested with a neurological assessment battery to discard possible motor and sensory deficits. Vaginal smears were taken in order to determine the stage of the estrous cycle. Sampling of the estrous cycle began exactly one week after neurological assessment battery to recover the baseline brain activity level and to avoid biased results. Vaginal epithelial cells were observed using an optical microscope (OLYMPUS, BH-2 model, Japan). The samples were taken just once a day, always at the same time for each subject (between 11:00 a.m.-1:00 p.m.). Males were manipulated in the same way as females in order to avoid stress differences. Rats were divided into three groups: male rats (*male group, n* = 8), rats in the estrus phase (*estrus group, n* = 8) and animals in the diestrus phase on day 1 (*diestrus group, n* = 8). When the females were in the desired estrous cycle phase, they were decapitated. The protocol used was the same described by Arias et al. (Arias et al., 2010). Brains were removed, frozen rapidly in N-methylbutane (Sigma-Aldrich, Madrid, Spain) and stored at -40 ºC until processing with quantitative C.O. histochemistry.

Instruments

Quantification of C.O. histochemical staining intensity was done by densitometric analysis, using a computer-assisted image analysis workstation (MCID, Interfocus Imaging Ltd., Linton, England) made up of a high precision illuminator, a digital camera and a computer with specific image analysis software. The mean optical density (OD) of each region was measured on bilateral structures, using three consecutive sections in each subject. In each section, four non-overlapping readings were taken, using a square-shaped sampling window that was adjusted for each region size. A total of twelve measurements were taken per region by an investigator blind to the groups. These measurements were averaged to obtain one mean per region for each animal. OD values were then converted to C.O. activity units, determined by the enzymatic activity of the standards measured spectrophotometrically (González-Lima & Cada, 1994).

The regions of interest were anatomically defined according to Paxinos and Watson´s atlas (2005). The regions of interest and the distance in mm of the regions counted from bregma was: +3.20 mm for the infralimbic cortex (IL), prelimbic cortex (PL), the cingulate cortex (CG); -1.40 for the anterodorsal thalamus (ADT), the anteroventral thalamus (AVT) and the mediodorsal thalamus (MDT); + 1.20 mm for the parietal cortex (Par); -1.20 mm for the CA1, CA3 and the dentate gyrus (DG) subfields of the dorsal hippocampus; -4.80 for ventral hippocampus; +4.52 mm for the supramammillary nucleus (SuM), the medial mammillary nucleus (MM), the medial lateral mammillary nucleus (ML) and the lateral mammillary nucleus (LM).

Data analysis

Group differences in C.O. activity measured in each brain region were evaluated by one-way ANOVA (factor: group). A p value <.05 was considered statistically significant. Post -hoc multiple comparisons analyses were carried out using Tukey's test.

In order to explore the intrahemispheric activity within each group, a one-way ANOVA (factor: group) was performed. A p value <.05 was considered as statistically significant. Data were analysed with Sigma-Stat 3.5 (Systat Software, Chicago, USA) and SigmaPlot 11.0 (Systat Software, Chicago, USA).

It is known that training experience in spatial learning could be manifested as neural changes in functional connectivity (Shao & Dongsheng, 1995; Fidalgo, Conejo, González-Pardo, & Arias, 2011) so we also wanted to check whether these changes existed at basal levels. The functional relationships among the regional brain activity data were analysed in terms of pairwise correlations within each experimental group. For the interrregional correlation analysis, Pearson´s product-moment correlations between pairs of brain regions in each experimental group were computed. In addition, in order to avoid errors due to an excessive number of significant correlations using small sample sizes, a "jackknife" procedure was used (Shao & Dongsheng, 1995). This procedure is based on the calculation of all possible pairwise correlations resulting from removing one subject each time, and taking into consideration only those correlations that remain significant (p<.05) across all possible interactions.

Results

C.O. activity levels in target regions

Mean regional C.O. activity measured in the different experimental groups is summarized in Table 1. Highly significant group × structures, $F(48, 546) = 5.085$, $p<.001$, were found in all structures analyzed. With regard to the prefrontal cortex, statistically significant differences in C.O. activity were found between males and the rest of experimental groups ($p<.05$; Tukey post-hoc tests) in the infralimbic cortex. Significant differences were found between diestrus and male groups in the prelimbic cortex ($p = .018$). However, no group differences were found in the cingulate cortex.

On the other hand, statistically significant differences in C.O. activity between males and the other experimental groups were found in dorsal hippocampal region: CA1 ($p<.05$), CA3 ($p<.05$) and dentate gyrus ($p<.05$). C.O. activity measured in the estrus group was significantly higher as compared with the male and diestrus groups in all hippocampal subfields; results were similar after comparing C.O. activity between diestrus females and the males in CA1 ($p = .002$), CA3 ($p = .029$) and dentate gyrus ($p<.001$). However, no significant C.O. activity differences between diestrus and male groups were found in the stratum radiatum (sr) of the CA1 and CA3 areas, the stratum lucidum (sl) and the stratum lacunosum-moleculare (slm) of the CA3 area and the polymorphic (pol) and molecular (ml) layers of the dentate gyrus. The rest of layers, such as stratum oriens (so) and granule cell layer (gcl) showed the same pattern as the subfields of the hippocampus.

Moreover, C.O. activity was statistically significant between groups in the CA1 area of the ventral hippocampus and CA3 area, showing higher C.O. activity in the estrus group as compared to the male group in CA1 ($p<.001$) and CA3 ($p<.001$) and in the diestrus group as compared to the male group in CA1 ($p<.001$) and CA3 ($p<.001$) (Table 1, # p<.05 significant difference between the estrus and male groups. + p<.05 significant difference between the estrus and diestrus groups. * p<.05 significant difference in the diestrus group as compared to the males. Data represent mean ± SEM).

Significant differences were found in all the thalamic regions between groups: ADT, AVT and MDT. Differences were found between the estrus group compared to the males in AVT ($p = .04$) and MDT ($p = .029$); the same was found between diestrus females and the male group in ADT ($p<.001$), AVT ($p<.001$) and MDT ($p<.001$); in addition, differences were found in females between diestrus and estrus groups in ADT ($p = .05$).

As regards to the mammillary bodies, C.O. activity was statistically significant between groups in the supramammillar nucleus ($p<.05$), the medial ($p<.05$), the medial lateral ($p<.05$) and lateral nucleus ($p<.05$). Higher C.O. activity was found in the estrus group than in the male group in suprammamilar ($p<.001$), medial ($p<.001$), medial lateral ($p<.001$) and lateral areas ($p<.001$). The same significant differences were found between the diestrus group and the male group in suprammamilar ($p<.001$), medial ($p<.001$), medial lateral ($p<.001$) and lateral ($p<.001$). Statistically significant differences were found in the estrus group as compared to the diestrus group in suprammamilar ($p = .008$), medial ($p<.001$), medial lateral ($p<.001$) and lateral ($p = .001$).

Table 1
C.O. activity measured in the selected brain regions in estrus, diestrus and male groups

Brain region	n	Estrus	n	Diestrus	n	Males
Prefrontal cortex						
Infralimbic	8	21.1±0.8$^{\#}$	7	23.3±0.3*	8	16.4±0.7
Prelimbic	8	20.7±0.7	8	22.4±0.7*	8	17.3±0.8
Cingulate cortex	8	21.2±0.5	7	22.0±0.7	8	17.8±1.0
Dorsal hippocampus						
CA1 area	8	28.0±0.9$^{\#+}$	8	23.6±3.6*	8	17.5±0.5
so	8	25.8±1.2$^{\#+}$	8	19.4±0.4*	8	15.4±0.7
sr	8	27.4±1.1$^{\#+}$	8	18.4±0.9	8	14.6±0.7
slm	8	40.1±1.2$^{\#+}$	8	26.8±1.1*	8	23.0±0.7
CA3 area	8	25.9±1.0$^{\#+}$	8	21.5±3.2*	8	16.9±0.6
so	8	34.9±1.4$^{\#+}$	8	23.2±0.8*	8	19.7±0.9
sl	8	23.8±1.0$^{\#+}$	8	17.6±0.5	8	14.5±0.7
sr	8	28.7±1.0$^{\#+}$	8	19.6±0.6	8	16.9±0.8
slm	8	19.2±1.0$^{\#+}$	8	13.8±0.7	8	11.5±0.6
Dentate gyrus	8	41.0±1.6$^{\#+}$	8	32.4±5.2*	8	23.4±0.8
pol	8	26.3±1.4$^{\#+}$	8	16.7±0.6	8	13.5±0.4
gcl	8	36.1±1.4$^{\#+}$	8	23.7±0.6*	8	19.7±0.5
ml	8	41.1±1.5$^{\#+}$	8	29.1±0.7	8	24.6±0.7
Ventral hippocampus						
CA1 area	8	28.7±1.1$^{\#}$	8	27.1±0.8*	8	19.1±0.9
CA3 area	8	30.4±0.7$^{\#}$	8	26.9±0.6*	8	20.1±1.0
Dentate gyrus	8	22.8±0.9	8	23.6±0.9	8	20.8±0.5
Thalamus						
Anterodorsal	8	34.3±0.6$^{\#+}$	8	38.5±1.0	8	31.1±1.3
Anteroventral	8	27.0±0.4$^{\#}$	8	30.4±0.8*	8	22.7±0.9
Mediodorsal	8	23.0±0.4$^{\#}$	8	24.9±1.0*	8	18.4±0.7
Mammilary bodies						
Supramammilar	8	26.2±1.4$^{\#+}$	8	20.8±1.0*	8	13.9±0.5
Medial medial	8	34.0±1.2$^{\#+}$	8	27.4±1.0*	8	19.9±0.8
Medial medial lateral	8	27.8±1.1$^{\#+}$	8	21.0±0.7*	8	14.0±0.7
Lateral	8	35.7±1.3$^{\#+}$	8	30.3±1.0*	8	23.0±0.5

Study of intragroup functional brain asymmetry

No significant differences in mean regional C.O. activity were found between the right and left hemispheres in any of the groups (Table 2, #p<.05 significant difference between the estrus and male groups. +p<.05 significant difference between the estrus and diestrus groups. *p<.05 significant difference the diestrus compared to the males. Data represent mean ± SEM).

Significant effects of group were found in the infralimbic cortex, $F(2, 40) = 55.022$, $p<.001$, with significant C.O. activity differences between the diestrus and male groups ($p<.001$; Tukey post-hoc tests), the diestrus and estrus groups ($p = .009$) and the estrus and male groups ($p<.001$) in the left hemisphere. The right hemisphere showed C.O. activity differences between the males and the rest of experimental groups ($p<.001$).

Significant group differences were found in the prelimbic, $F(2, 39) = 21.888$, $p<.001$, and cingulate cortex, $F(2, 40) = 14.902$, $p<.001$, with differences between the males and the rest of the experimental groups ($p<.005$) in both hemispheres.

Regarding the dorsal hippocampus, group differences were found in CA1 subfield, $F(2, 40) = 63.021$, $p<.001$, CA3 subfield, $F(2, 40) = 56.868$, $p<.001$, and the dentate gyrus, $F(2, 40) = 55.116$, $p<.001$. Significant differences in C.O. activity were found between the males and the rest of the groups ($p<.05$) in both the right and left hemispheres. Furthermore, the layers of the CA1 subfield in the left hippocampus showed the same pattern of differences in C.O. activity. However, the so and slm layers of the CA3 subfield showed no C.O. activity differences between the diestrus and male groups. The layers of the dentate gyrus showed only differences between the estrus and diestrus groups in the gcl and ml layers. The left and the right hemisphere showed the same pattern in the slm of the CA1 area and in the sl and the sr of the CA3 area.

The ventral hippocampus showed significant group × hemisphere interactions in CA1 subfield, $F(2, 36) = 3.938$, $p = .028$, and significant group differences in the CA3 subfield, $F(2, 36) = 75.928$, $p<.001$, and the dentate gyrus, $F(2, 36) = 4.127$, $p = .024$. Differences between the male group and the rest of the experimental groups were found in all subfields of the ventral hippocampus, except for the dentate gyrus, where no differences between groups were found.

On the other hand, the thalamus showed group differences in C.O. activity of the anterodorsal nucleus, $F(2, 42) = 24.053$, $p<.001$, the anteroventral nucleus, $F(2, 42) = 51.821$, $p < .001$, and the mediodorsal nucleus, $F(2, 42) = 36.944$, $p<.001$. The anteroventral thalamic nucleus showed significant differences between the diestrus and the rest of the experimental groups and between the estrus and male groups ($p<.05$) in both hemispheres whereas the anterodorsal nucleus did not show significant differences between the estrus and male groups in the left hemisphere. Lastly, the mediodorsal thalamic nucleus showed differences between the males and the rest of the experimental groups ($p<.05$).

Regarding the mammillary bodies, group differences were found in medial lateral nucleus , $F(2, 41) = 238.160$, $p<.001$, and the lateral nucleus, $F(2, 42) = 33.523$, $p<.001$. In addition, C.O. activity was significantly different between the estrus and the rest of the groups ($p<.05$) in the aforementioned mammillary nuclei in both the right and left hemispheres.

Table 2
C.O. activity measured in the right (RH) and left (LH) hemispheres of the selected brain regions in estrus, diestrus and male groups

Brain region	LH						RH					
	n	Estrus	n	Diestrus	n	Male	n	Estrus	n	Diestrus	n	Male
Prefrontal cortex												
Infralimbic region	8	20.8±0.8$^{\#}$	7	23.8±0.3^{*+}	8	16.7±0.8	8	21.4±0.8$^{\#}$	7	22.8±0.3*	8	16.0±0.7
Prelimbic region	8	20.8±0.7$^{\#}$	7	23.3±0.8*	8	17.3±0.8	8	20.5±0.8$^{\#}$	6	21.6±0.5*	8	17.3±1.0
Cingulate region	8	21.4±0.5$^{\#}$	7	21.9±0.8*	8	17.7±1.0	8	21.0±0.5$^{\#}$	7	22.1±0.7*	8	17.9±1.1
Dorsal hippocampus												
CA1 area	8	27.4±0.8$^{\#}$	7	26.5±1.6*	8	17.1±0.4	8	28.5±1.1$^{\#}$	7	27.5±1.5*	8	18.0±0.6
so	8	25.6±1.2$^{\#+}$	8	19.5±0.6*	8	15.0±0.7	5	26.0±1.2$^{\#+}$	8	19.4±0.5*	8	15.8±0.8
sr	8	26.8±0.9$^{\#+}$	8	17.8±0.9*	8	14.4±0.9	5	28.1±1.3$^{\#+}$	8	18.9±1.0*	8	14.7±0.7
slm	8	39.3±1.4$^{\#+}$	8	26.9±1.1*	8	22.6±1.0	5	40.9±1.3$^{\#+}$	8	26.9±1.2	8	23.3±0.5
CA3 area	8	25.4±1.1$^{\#}$	7	24.1±1.2*	8	16.4±0.6	8	26.5±1.0$^{\#}$	7	24.9±0.9*	8	17.3±0.7
so	8	34.5±1.6$^{\#+}$	7	23.0±0.9	8	19.4±1.0	8	35.3±1.3$^{\#+}$	7	23.4±0.8	8	20.0±0.8
sl	8	23.0±1.0$^{\#+}$	7	17.5±0.6*	8	13.9±0.8	8	24.6±1.0$^{\#+}$	7	17.7±0.5	8	15.0±0.6
sr	8	27.9±1.3$^{\#+}$	7	19.5±0.7*	8	16.3±1.0	8	29.4±0.9$^{\#+}$	7	19.6±0.6	8	17.5±0.7
slm	8	18.9±1.1$^{\#+}$	7	13.7±0.6	8	11.1±0.7	8	19.6±0.9$^{\#+}$	7	13.9±0.7*	8	11.9±0.6
Dentate gyrus	8	41.2±1.5$^{\#}$	7	36.9±2.9*	8	23.6±0.8	8	40.8±1.7$^{\#}$	7	37.2±2.5*	8	23.1±0.9
pol	8	26.0±1.7$^{\#}$	7	16.7±0.6*	8	13.3±0.4	8	26.6±1.1$^{\#}$	7	16.6±0.8*	8	13.7±0.4
gcl	8	36.4±1.5$^{\#+}$	7	23.3±0.5*	8	19.7±0.6	8	35.9±1.5$^{\#+}$	7	24.1±0.7*	8	19.8±0.5
ml	8	41.3±1.5$^{\#+}$	7	29.5±0.8*	8	24.7±0.8	8	40.9±1.5$^{\#+}$	7	28.7±0.7*	8	24.4±0.7
Ventral hippocampus												
CA1 area	8	27.8±0.9$^{\#}$	8	26.4±1.0*	8	19.8±0.9	5	31.3±1.3$^{\#}$	8	27.8±0.8*	8	18.5±0.9
CA3 area	8	30.1±0.9$^{\#}$	8	27.3±0.8*	8	20.1±1.1	5	30.8±0.9$^{\#}$	8	26.5±0.5*	8	20.2±1.0
Dentate gyrus	8	22.7±1.1	8	24.3±1.2	8	20.7±0.4	5	23.8±1.0	8	22.8±0.9	8	21.0±1.0
Thalamus												
Anterodorsal n.	8	34.0±0.8		38.3±0.9^{*+}	8	31.5±1.4	8	34.6±0.5$^{\#}$	8	38.8±1.2^{*+}	8	30.6±1.4
Anteroventral n.	8	26.6±0.4$^{\#}$		30.4±0.8^{*+}	8	22.3±0.7	8	27.4±0.4$^{\#}$	8	30.4±0.9^{*+}	8	23.0±1.1
Mediodorsal n.	8	22.7±0.5$^{\#}$		24.9±1.2*	8	18.6±0.8	8	23.3±0.5$^{\#}$	8	25.0±0.9*	8	18.2±0.7
Mammillary bodies												
Medial n.	8	27.2±1.2$^{\#+}$	8	20.7±0.7	8	14.4±0.8	8	28.51.1$^{\#+}$	8	21.4±0.7	8	13.5±0.7
Lateral n.	8	35.8±1.4$^{\#+}$	8	29.6±1.2	8	23.0±0.8	8	36.3±1.2$^{\#+}$	8	31.1±0.9	8	23.0±0.5

Interregional within-group correlations of C.O. activity

Female rats in estrus phase. A high cross-correlation was found between prelimbic cortex and infralimbic cortex (r = -.97, p<.001), between mediodorsal thalamus and dentate gyrus of the ventral hippocampus (r = .85, p = .01), between the medial lateral area and the medial area of the mammillary bodies (r = .96, p<.001), as well as between medial area of the mammillary bodies and dentate gyrus of the ventral hippocampus (r = - .86, p = .01) (Figure 1, Solid and dotted lines represent, respectively, highly positive and negative pair-wise Pearson's correlations [r > .8, p≤.02]).

Female rats in diestrus phase. A high cross-correlation was observed between prelimbic cortex and cingulate cortex (r = .86, p = .01), between the CA1 subfield and CA3 subfield of the dorsal hippocampus (r = .99, p<.001), between the CA1 and the dentate gyrus of the dorsal hippocampus (r = .98, p<.001), and between the dentate gyrus and CA3 subfield (r = .97, p<.001) as well as between the lateral area and the medial area of the mammillary bodies (r = .81, p = .02) (Figure 1).

Male rats. A high cross-correlation was found between the CA1 subfield and CA3 subfield of the dorsal hippocampus (r = .92, p = .001), between the CA1 subfield of the ventral hippocampus and medial area of the mammillary bodies (r = -.83, p = .01), between the medial lateral area of the mammillary bodies and the anterodorsal thalamus (r = -.81, p = .02) as well as between the anteroventral and mediodorsal thalamus (r = .95, p<.001) (Figure 1).

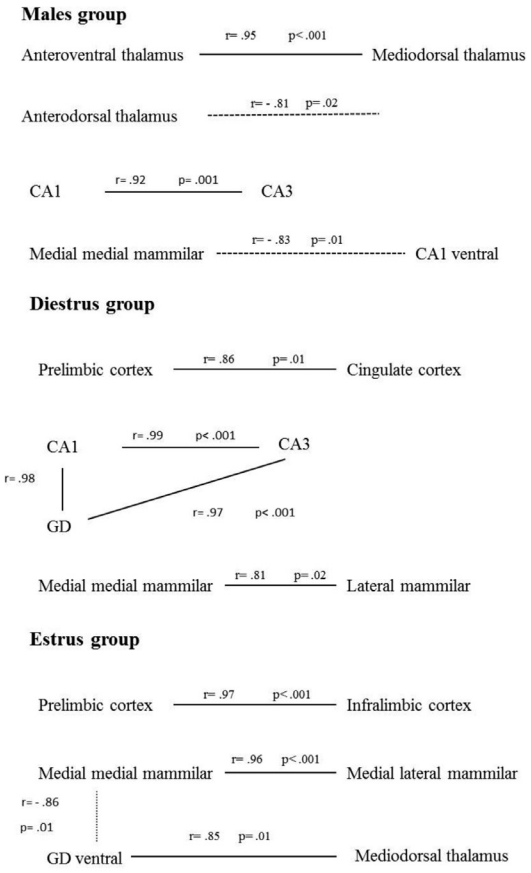

Figure 1. *Schematic diagram showing the significant interregional correlations of C.O. activity calculated in the studies groups (male, estrus and diestrus)*

Discussion

This study demonstrated the influence of fluctuation of ovarian hormones on the glucose metabolism in brain areas involved in emotion, a function also associated with the regions studied herein. In our experiment, the PFC and the hippocampus were chosen mainly for their relationship with basic brain functions as memory and learning (Conejo et al., 2007). Among these regions, a significant role has been attributed to the dorsal hippocampus in relation to spatial memory (Méndez-López, Méndez, López, & Arias, 2009). Therefore, we think that it is interesting to analyze this region in more detail by quantification of C.O. activity within dorsal hippocampal layers. To our knowledge, this is the first study that analyzes the neuronal oxidative metabolism by C.O. histochemistry across the estrous cycle in hippocampus layers, which allowed us to determine the distribution C.O. activity within dorsal hippocampus. In this study, we found that C.O. activity in the dorsal hippocampus is not homogeneous because different C.O. activity levels were measured in their layers. This variability could be due to fluctuations in endogenous ovarian hormones, which have been shown to be able to alter dendritic spine density of pyramidal neurons in the hippocampus subfields (CA1 and CA3) of adult female rats (Parducz & García-Segura, 1993).

The dopaminergic system is known to influence the growth and differentiation of mPFC pyramidal cell dendritic arbor (Kalsbeek, Matthijssen, & Uylings, 1989) as well as neuronal activity in this area (Thierry, Godbout, Mantz, & Glowinski, 1990), sexual dimorphism in the dopaminergic system could contribute to sex differences in the mPFC. Despite interactions between ovarian hormones and the dopamine system that innervates the mPFC, dendritic spine density and arborization in the anterior cingulate do not appear to be sensitive to differences in endogenous levels of ovarian hormones (Markham & Juraska, 2002). It may be that the cortex is simply not as sensitive to changing levels of ovarian hormones as the hippocampus. A similar pattern was found in mediodorsal nucleus of the thalamus, which was assumed to be the main nucleus with thalamocortical projections to the prefrontal cortex, although the prefrontal cortex is also connected to other thalamic nuclei (Uylings, Groenewegen, & Kolb, 2003).

According to these results, in the CA1 and CA3 area of the ventral hippocampus, differences between the different cycle stages studied and the males were found. It is known that estrogens can directly increase cytochrome oxidase (C.O.) activity in rat hippocampus in a few hours by increasing the C.O. levels of several catalytic subunits (Nilsen, Irwin, Gallaher, & Brinton, 2007) and by increasing oxygen consumption in brain mitochondria (Klinge, 2008). In contrast, the higher activity shown in these areas questions the proportional direct relationship between estrogen levels and brain metabolic activity. Therefore, estrogenic influence seems to be region-specific and different factors, such as ER levels of differences in anatomical connectivity, may explain this divergent result.

Similar results have been obtained in the mammillary bodies, which are connected via the fornix to the hippocampus (Blanco, Picón, Miranda, Begega, Conejo, & Arias, 2006). Indeed, recent studies have demonstrated two different neurochemical pathways in rat. The first originates from neurons in the lateral region of the supramammillary nucleus and it innervates the supragranular layer of the dorsal dentate gyrus and, to a much lesser extent, the ventral dentate gyrus, and the second pathway originates from neurons

in the most posterior and medial part of the supramammillar and innervates exclusively the inner molecular layer of the ventral dentate gyrus and the CA2-CA3a pyramidal cell layer of the hippocampus (Soussi, Zhang, Tahtakran, Houser, & Esclapez, 2010). It is important to point out the lower activity in males than females not only in the mammillary bodies, as shown by other authors (Blanco et al., 2006), although not in all structures analyzed. However, in contrast to Blanco et al. (2006), differences in C.O. activity in female mammillary bodies were not evident between the phases of the estrous cycle studied. Our study has improved the technique used and probably was responsible for finding differences between estrus and diestrus. Indeed, the pattern of C.O. activity in the medial and lateral mammillary bodies is the same as has been shown in that study.

All these circuits are well reflected by the correlation coefficients, where relationships between ventral hippocampus, thalamus and mammillary bodies are involved in males and estrus females, whereas prefrontal cortex did not correlate with other structures in diestrus and estrus females. Finally, we would like to point out the different involvement of the dorsal hippocampus in males, where CA1 and CA3 subfields are directly connected, whereas in diestrus females, both subfields are in relation to dentate gyrus agreeing a small circuit.

On the other hand, brain lateralization may be conditioned by age, gender, life conditions and circulating hormone levels. Interestingly, no differences in neuronal metabolic activity between the right and left hemispheres were found in the groups.

As regards the study of intergroup functional asymmetry, higher left hemisphere C.O. activity was found in the infralimbic cortex (diestrus and male groups) and the prelimbic cortex in all groups. The same left predominance was observed in the dentate gyrus of estrus females and in males and in CA3 and the dentate gyrus of the ventral hippocampus in diestrus females. In contrast, all groups had higher right-hemisphere neuronal metabolic activity in the remaining structures. These data are also supported by differences in overall C.O. activity of the prefrontal cortex between groups, where it was found that both right and left hemisphere of all prefrontal cortex regions showed higher C.O. activity in diestrus phase than in the rest of the groups. In contrast, except for the dentate gyrus and thalamus, both left and right hemispheres of all areas were more activated in the estrus than in the diestrus stage. These differences in C.O. could explain previous works in which sex differences in learning strategies were found in rodents (Méndez-López et al., 2009) and could support the findings of Blokland, Rutten and Prickaerts (2006), who found a sex difference in the place learning strategy favouring male Wistar rats in the Morris water maze. Therefore, the possible mechanism of action of estrogens in the brain seems to be very complex, and more studies are required in order to address this question.

Acknowledgements

This work was supported by grant PSI2010-19348 (Spanish Ministry of Education and Science and Innovation and European Regional Development Fund), AP/6977-2009 (FMMA), MEC AP2009-1714 to N. Arias and J. Morán has been subsidized by the Principality of Asturias Government.

References

Afonso, D., Santana, C., & Rodríguez, M. (1993). Neonatal lateralization of behavior and brain dopaminergic asymmetry. *Brain Research Bulletin*, *32*(1), 11-16.

Arias, N., Álvarez, C., Conejo, N., González-Pardo, H., & Arias, J.L. (2010). Estrous cycle and sex as regulating factors of baseline brain oxidative metabolism and behavior. *Revista Iberoamericana de Psicología y Salud, 1*(1), 3-16.

Blanco, E., Picón, I.M., Miranda, R., Begega, A., Conejo, N.M., & Arias, J.L. (2006). Astroglial distribution and sexual differences in neural metabolism in mammillary bodies. *Neuroscience Letters*, *395*(1), 82-86.

Blokland, A., Rutten, K., & Prickaerts, J. (2006). Analysis of spatial orientation strategies of male and female Wistar rats in a Morris waster escape task. *Behavioral Brain Research*, *171*(2), 216-224.

Chai, X.J., & Jacobs, L.F. (2010). Effects of cue types on sex differences in human spatial memory. *Behavioral Brain Research*, *208*(2), 336-342.

Chen, J.Q., Yager, J.D., & Russo, J. (2005). Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications. *Biochimica et Biophysica Acta*, *1746*(1), 1-17.

Conejo, N.M., González-Pardo, H., Vallejo, G., & Arias, J.L. (2004). Involvement of the mammillary bodies in spatial working memory revealed by cytochrome oxidase activity. *Brain Research*, *1011*(1), 107-114.

Conejo, N.M., González-Pardo, H., Vallejo, G., & Arias, J.L. (2007). Changes in brain oxidative metabolism induced by water maze training. *Neuroscience*, *145*(2), 403-412.

Conrad, C.D., Grote, K.A., Hobbs, R.J., & Ferayorni, A. (2003). Sex differences in spatial and non-spatial Y-maze performance after chronic stress. *Neurobiology of Learning and Memory*, *79*(1), 32-40.

Fidalgo, C., Conejo, N.M., González-Pardo, H., & Arias, J.L. (2011). Cortico-limbic-striatal contribution after response and reversal learning: A metabolic mapping study. *Brain Research*, *1368*, 143-150.

González-Lima, F., & Cada, A. (1994). Cytochrome oxidase activity in the auditory system of the mouse: A qualitative and quantitative histochemical study. *Neuroscience*, *63*(2), 559-578.

Kalsbeek, A., Matthijssen, M.A., & Uylings, H.B. (1989). Morphometric analysis of prefrontal cortical development following neonatal lesioning of the dopaminergic mesocortical projection. *Experimental Brain Research*, *78*(2), 279-289.

Klinge, C.M. (2008). Estrogenic control of mitochondrial function and biogenesis. *Journal of Cellular Biochemistry*, *105*(6), 1342-1351.

Koike, S., Sakai, M., & Muramatsu, M. (1987). Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Research*, *15*(6), 2499-2513.

Kristofiková, Z., Kozmiková, I., Hovorková, P., Rícný, J., Zach, P., Majer, E., et al. (2008). Lateralization of hippocampal nitric oxide mediator system in people with Alzheimer disease, multi-infarct dementia and schizophrenia. *Neurochemistry International*, *53*(5), 118-125.

Kritzer, M.F. (2002). Regional, laminar, and cellular distribution of immunoreactivity for ER alpha and ER beta in the cerebral cortex of hormonally intact, adult male and female rats. *Cerebral Cortex*, *12*(2), 116-128.

Kuiper, G.G., Enmark, E., Pelto-Huikkom, M., Nilsson, S., & Gustafsson, J.A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(12), 5925-5930.

Markham, J.A., & Juraska, J.M. (2002). Aging and sex influence the anatomy of the rat anterior cingulate cortex. *Neurobiology of Aging*, *23*(4), 579-588.

Méndez-López, M., Méndez, M., López, L., & Arias, J.L. (2009). Spatial working memory learning in young male and female rats: Involvement of different limbic system regions revealed by cytochrome oxidase activity. *Neuroscience Research*, *65*(1), 28-34.

Nilsen, J., Irwin, R.W., Gallaher, T.K., & Brinton, R.D. (2007). Estradiol in vivo regulation of brain mitochondrial proteome. *Journal of Neuroscience*, *27*(51), 14069-14077.

Oke, A., Lewis, R., & Adams, R.N. (1980). Hemispheric asymmetry of norepinephrine distribution in rat thalamus. *Brain Research*, *188*(1), 269-272.

Parducz, A., & García-Segura, L.M. (1993). Sexual differences in the synaptic connectivity in the rat dentate gyrus. *Neuroscience Letters*, *161*(1), 53-56.

Paxinos, G., & Watson, C.H. (2005). *The rat brain in stereotaxic coordinates-the new coronal set*. 5th ed. London, UK: Elsevier Academic Press.

Rubio, S., Miranda, R., Cuesta, M., Begega, A., Santín, L.J., & Arias, J.L. (1999). Active avoidance conditioning in rats: Absence of sex difference and estrous effect. *Psicothema*, *11*(3), 655-661.

Samara, A., Vougas, K., Papadopoulou, A., Anastasiadou, E., Baloyanni, N., Paronis, E., et al. (2011). Proteomics reveal rat hippocampal lateral asymmetry. *Hippocampus*, *21*(1), 108-119.

Sanders, G., & Wenmoth, D. (1998). Verbal and music dichotic listening tasks reveal variations in functional cerebral asymmetry across the menstrual cycle that are phase and task dependent. *Neuropsychologia*, *36*(9), 869-874.

Sashkov, V.A., Sel'verova, N.B., Morenkov, É.D., & Ermakova, I.V. (2010). Sex-related peculiarities of conditioned reflex activity and dynamics of sex steroids in the brain. *Zhurnal Evoliutsionnoi Biokhimii i Fiziologii*, *46*(4), 304-310.

Saucier, D.M., Shultz, S.R., Keller, A.J., Cook, C.M., & Binsted, G. (2008). Sex differences in object location memory and spatial navigation in Long-Evans rats. *Animal Cognition*, *11*(1), 129-137.

Shao, J., & Dongsheng, T. (1995). *The jackknife and bootstrap*. New York: Springer-Verlag.

Soussi, R., Zhang, N., Tahtakran, S., Houser, C.R., & Esclapez, M. (2010). Heterogeneity of the supramammillary-hippocampal pathways: Evidence for a unique GABAergic neurotransmitter phenotype and regional differences. *European Journal of Neuroscience*, *32*(5), 771-785.

Thierry, A.M., Godbout, R., Mantz, J., & Glowinski, J. (1990). Influence of the ascending monoaminergic systems on the activity of the rat prefrontal cortex. *Progress in Brain Research*, *85*, 357-364.

Uylings, H.B., Groenewegen, H.J., & Kolb, B. (2003). Do rats have a prefrontal cortex? *Behavioral Brain Research*, *146*(1-2), 3-17.

Wisniewski, A.B. (1998). Sexually-dimorphic patterns of cortical asymmetry and the role for sex steroid hormones in determining cortical patterns of lateralization. *Psychoneuroendocrinology*, *23*(5), 519-547.