Effects of Estradiol on 5-HT\textsubscript{5A} and 5-HT\textsubscript{2C} Receptor Immunolabeling in Rat Hippocampus

Laura Cristina Berumen\textsuperscript{1}, Marco Antonio Sánchez-Ramos\textsuperscript{2}, Martín García-Servín\textsuperscript{3}, Ataulfo Martínez-Torres\textsuperscript{3}, Angelina Rodríguez\textsuperscript{1} and Guadalupe García-Alcocer\textsuperscript{1}

1. Facultad de Química, Universidad Autónoma de Querétaro, Centro Universitario, Querétaro 76010, México
2. Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro, Campus Juriquilla, Juriquilla Querétaro 76230, México
3. Department of Cellular and Molecular Neurobiology, Instituto de Neurobiología, Universidad Nacional Autónoma de México, Campus Juriquilla, Juriquilla Querétaro 76230, México

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Abstract: Steroid hormones participate in the modulation of serotonergic transmission, including the regulation of synthetic and metabolic enzyme production, as well as receptor and transporter activity. The changes of 5-HT\textsubscript{5A} and 5-HT\textsubscript{2C} immunolabeling induced by steroids in the hippocampus of ovariectomized rats were studied in this work. Densitometric analysis in rat hippocampi were carried out for adjacent brain coronal immunolabeled sections after treatment with subcutaneous injections of vehicle, estradiol, progesterone or the combination of both steroids in ovariectomized rats. Exposure to estradiol and the combination of estradiol and progesterone significantly reduced the 5-HT\textsubscript{5A}-like immunosignal in the CA1 region while progesterone did not induce changes. On the other hand, exposure to the combination of estradiol and progesterone or estradiol alone increased the 5-HT\textsubscript{2C} immunosignal in the same region. These results indicate that estradiol is involved in the discrete regulation of serotonin receptors 5-HT\textsubscript{5A} and 5-HT\textsubscript{2C} in rat hippocampus.

Key words: Serotonin receptor, 5-HT\textsubscript{5A}, 5-HT\textsubscript{2C}, hippocampus, estradiol, progesterone.

1. Introduction

Steroid hormones, which include estrogens, progestins, androgens, glucocorticoids and mineralocorticoids, have ligand-inducible transcription factors-mediated actions; they also have several non-genomic effects that modify the transcriptional activity of different genes. These effects include essential roles played by steroid hormones in: brain differentiation, neural plasticity and neurotransmission [1-3].

Steroid hormones have modulatory effects on the synthesis and release of several neurotransmitters such as serotonin [4, 5] and affect serotonergic transmission in some extent by regulating the density of receptors in neuronal plasma membranes [6-8]. The 5-HT\textsubscript{5A} receptor, a G protein-coupled metabotropic receptor, is involved in the modulation of exploratory behavior and in certain LSD-psychoactive effects; it is expressed in the brain, with the highest concentrations found in the hippocampus [9-11]. Activation of 5-HT\textsubscript{5A} receptors inhibits the production of cAMP but its precise metabolic cascade has not been fully elucidated [12-14].

On the other hand, 5-HT\textsubscript{2C} receptors are heterogeneously distributed in the brain with the highest levels in the choroid plexus [15, 16]; they are involved in the control of appetite and tonic inhibition of neuronal network excitability [17, 18]. The 5-HT\textsubscript{2C} receptor regulates a number of effectors through PLC-, Ca\textsuperscript{2+}-, or PKC-dependent mechanisms [14].

Serotonergic pathways innervate the hippocampus and other limbic regions where they play an essential role in mood control and memory [17, 19]. Taking into consideration the known serotonergic activation in the

Corresponding author: Laura Cristina Berumen, Ph.D., professor, research fields: neurobiology, neuroendocrinology. E-mail: berumen@uaq.mx.
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hippocampus and the modulation of a number of serotonin receptors by steroids, tests were conducted to ascertain if estradiol and progesterone alter the expression and location of 5-HT\textsubscript{5A} receptors in the rat hippocampus compared to 5-HT\textsubscript{2C}.

2. Materials and Methods

Adult female Sprague-Dawley rats (200-250 g) were maintained with food and water ad libitum in 12 h/12 h light-dark cycles. Each experimental group consisted of five rats ovariectomized under anesthesia (80 mg/kg ketamine + 6 mg/kg xylazine) between 9-12 h in the morning of diestrus. Administration of all treatments was carried out between 9-10 h in the morning of days 1 through 5 after surgery using a single daily subcutaneous injection. The control group of rats was administered only vehicle (100 \(\mu\)L corn oil subcutaneous injection). A group of ovariectomized rats was treated with 50 \(\mu\)g/kg body weight of estradiol benzoate (EB). Another group was treated with 7.5 mg/kg of progesterone (P) and a last group was injected with the combination of EB and P. After treatments rats were anesthesized with sodium pentobarbital (40 mg/kg) and decapitated between 9-12 h the next day following the treatment. Brains were fixed in 4% paraformaldehyde-PBS and coronal sections of 12 \(\mu\)m were made in a Leica CM 1850 cryostat, then thaw mounted on superfrost slides [20].

Immunohistochemistry was performed with specific antibodies used to determine the distribution of the receptors: 1) an anti 5-HT\textsubscript{5A} receptor (Sigma; 1:500); and 2) an anti 5-HT\textsubscript{2C} (Santa Cruz Biotechnologies; 1:500). The slices were incubated overnight at room temperature with the primary antibody; after rinsing, they were incubated with the biotin-conjugated goat anti-rabbit antibody (Chemicon) for two hours at room temperature and then with Vector ABC system. Finally, 3,3’-diaminobenzidine (DAB) and hydrogen peroxide were used for color development. Digitized images of nine sections for each rat were analyzed using densitometry (Axiovision, Zeiss). One-way ANOVA and Tukey tests were used for statistical analysis of differences between group means. All experiments were conducted according to the international guidelines of the care and use of experimental animals.

3. Results

Immunodetection of 5-HT\textsubscript{5A} receptor in the hippocampus of untreated rats revealed that it is distributed in all regions. Rats treated with EB or EB + P showed an overall reduced staining for 5-HT\textsubscript{5A} (Fig. 1) in CA1 region. In contrast, P did not elicit significant changes compared to controls.

Densitometric analysis of the samples (Fig. 2) confirmed the observations made under the microscope, with a 11.4% reduction of intensity for 5-HT\textsubscript{5A} receptor-like

![Fig. 1 Coronal sections of rat brains stained by immunohistochemistry (DAB, H\textsubbox{2}O\textsubbox{2}) with an antibody against the 5-HT\textsubscript{5A} or 5-HT\textsubscript{2C} serotonin receptors.](image-url)

A, E: Ovariectomized rat control; B, F: Treated with 17\(\beta\)-estradiol; C, G: Treated with 17\(\beta\)-estradiol and progesterone; D, H: Treated with progesterone. Densitometry was performed for hippocampus. Scale bar: 1,000 \(\mu\)m.
immunosignal for EB treatment and 10.2% for EB + P treatment \((P < 0.01)\). No statistical difference for the 3.9% reduction of intensity in P treatment compared to control, and no statistical differences between EB and EB + P were noted.

The effects of EB and EB + P on 5-HT\(_{2C}\) receptors (Fig. 3) were the opposite to those on 5-HT\(_{5A}\) receptors; consistently, both treatments gave rise to higher levels of 5-HT\(_{2C}\) immunolabeling in CA1 (Fig. 1) compared to controls that showed 2.5% and 2% respectively, \(P < 0.01\) for EB + P. In contrast, rats treated with P alone did not differ from the control samples in that less than 1% reduction with no significant difference noted.

The densitometry values are arbitrary optical density units normalized against negative controls (no primary antibody). Estradiol (EB) and Estradiol + Progesterone (EB + P) decreased receptor expression with statistical significance \((\ast)\) compared to controls \((P < 0.01)\).

4. Discussion

The activity of different neurotransmitter systems can be modulated by steroid hormones. The effect of steroids over the serotonergic pathway has been widely studied by different means, although the partial agonism of classical drugs and ligands for the various serotonin receptors sometimes overlapping, makes it complex to analyze [21, 22]. In this work we found that estradiol changed the expression of 5-HT\(_{5A}\) and 5-HT\(_{2C}\) serotonin receptor subtypes. It has been reported that the administration of estrogen sharply reduced \([\text{H}]5\text{-HT}\) binding-sites in the hypothalamus and preoptic nucleus [7, 8, 23]; however the response is different for subtypes of serotonin receptor and brain areas. The effects of steroids have been studied in different areas of rat brain for several receptor subtypes, especially 5-HT\(_{1A}\) and 5-HT\(_{2A}\), but 5-HT\(_{5A}\) has received less attention. For example, estradiol reduced the 5-HT\(_{1A}\) receptor mRNA in the piriform cortex and the anterodorsal medial amygdale whereas it remained unaltered in the hippocampus and the prefrontal and cingulate cortex as well as in the dorsal nucleus of raphe [24, 25]. Furthermore, estradiol increased 5-HT\(_{2A}\) mRNA in several limbic regions of gonadectomized rats treated with estradiol or testosterone [26, 27] although no changes or reduced levels for 5-HT\(_{2C}\) mRNA were found for discrete areas of hippocampus [28].

It has been previously reported that the 5-HT\(_{5A}\) receptor undergoes developmental modifications and is a suitable candidate for fine-tuning regulation of the serotonergic system [20]. Changing conditions such as steroid levels may also exert different modulatory effects on the number and class of serotonin receptors present in the plasma membrane. In this project it was
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found that estradiol down-regulated 5-HT5A receptors. The results herein regarding this receptor were consistent with the down-regulation expected for the negative cAMP G-protein coupled receptor 5-HT1A although differences were found particularly in hippocampus [25]. On the other hand, progesterone action requires estrogen-priming in order to be effective [29]. No-changes were found with the subcutaneous high dosis administration of progesterone alone in these experiments. However, administering both estradiol and progesterone showed no statistical differences versus estradiol alone therefore it can be inferred that progesterone does not have a differential effect at this high acute dosis.

For the 5-HT2C receptor-like immunodetection there were found significant increases of signal noted after treatment with estradiol and estradiol plus progesterone which support the idea for specific cellular modifications in the serotonergic transmission. These results also correspond with the changes in PLC G-protein coupled serotonin receptor 5-HT2A [26] and 5-HT2C [30]. Nonetheless, it was found found that there were differences in receptor expression in hippocampus using subcutaneously implanted pellets [28] that might reflect differences in the dosages used and way of administration.

The different effects of estrogen and progesterone on the expression of serotonin receptors need to be further investigated in order to determine whether the effects are directly on the transcriptional regulation of the receptor protein, or reflect the contribution of the serotonin itself and its pathways. Although there are no recognizable canonical ERE in 5-HT5A sequence (ratCHR4:2707261-2716944) [31] and its 5’ and 3’ flanking regions (5 kbp), there are ERE-like motifs with different spacing regions between palindromic and direct repeats [32, 33], particularly one pentameric modified palindromic sequence with a four-bp spacer (CHR4:2717587-2717574) and the glucocorticoid receptor recognized sequence 3-bp spaced from an ERE-like motif (CHR4:2702313-2702299). This direct interaction needs to be tested, although it has been reported the estradiol induced expression for this receptor in rat anterior pituitary cell aggregates [34].

Moreover, the steroids may regulate receptor expression via other transcription factors, including cyclic AMP response element binding proteins. Estradiol causes phosphorylation of CREB in the CA1 region as well as in the CA3, which might be involved in the tissue-specific modification of serotonin receptors and differences found in in vitro experiments [1, 28, 35]; furthermore, pCREB immunolabeling is increased in hippocampus after estradiol administration as well as BDNF expression [36], which are involved in the expression of some serotonin receptors.

5. Conclusion

Administration of acute dosis of estradiol in ovariectomized rats down-regulates 5-HT5A and up-regulates 5-HT2C receptors in CA1 region of hippocampus, whereas progesterone alone does not induce significant changes in the expression of these receptors.

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