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Effects of tamoxifen on neuronal morphology, connectivity and biochemistry of hypothalamic ventromedial neurons: Impact on the modulators of sexual behavior

Susana I. Sá[a,b,*], Natércia Teixeira[c], Bruno M. Fonseca[c]

[a] Department of Biomedicine, Unit of Anatomy, Faculty of Medicine, University of Porto, Portugal
[b] Center for Health Technology and Services Research (CINTESIS), Faculty of Medicine, University of Porto, Portugal
[c] UCIBIO, REQUIMTE, Laboratory of Biochemistry, Department of Biological Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira, n. 228, 4050-313 Porto, Portugal

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ABSTRACT

Tamoxifen (TAM) is a selective estrogen receptor modulator, widely used in the treatment and prevention of estrogen-dependent breast cancer. Although with great clinical results, women on TAM therapy still report several side effects, such as sexual dysfunction, which impairs quality of life. The anatomo-functional substrates of the human sexual behavior are still unknown; however, these same substrates are very well characterized in the rodent female sexual behavior, which has advantage of being a very simple reflexive response, dependent on the activation of estrogen receptors (ERs) in the ventrolateral division of the hypothalamic ventromedial nucleus (VMNvl). In fact, in the female rodent, the sexual behavior is triggered by increasing circulation levels of estradiol that changes the nucleus neurochemistry and modulates its intricate neuronal network. Therefore, we considered of notice the examination of the possible neurochemical alterations and the synaptic plasticity impairment in VMNvl neurons of estradiol-primed female rats treated with TAM that may be in the basis of this neurological disorder. Accordingly, we used stereological and biochemical methods to study the action of TAM in axospinous and axodendritic synaptic plasticity and on ER expression. The administration of TAM changed the VMNvl neurochemistry by reducing ERα mRNA and increasing ERβ mRNA expression. Furthermore, present results show that TAM induced neuronal atrophy and reduced synaptic connectivity, favoring electrical inactivity. These data suggest that these cellular and molecular changes may be a possible neuronal mechanism of TAM action in the disruption of the VMNvl network, leading to the development of behavioral disorders.

1. Introduction

Tamoxifen (TAM) is a drug widely used in the prevention and treatment of estrogen-dependent breast cancer (Davies et al., 2013; Jordan, 2001; Yang et al., 2013). Although TAM therapy has shown very good results in breast cancer treatment, women treated with TAM experience several side effects, such as sexual dysfunction, which may affected their quality of live and encourage nonadherence to the treatment (Azim et al., 2016; Mortimer et al., 1999). The anatomo-functional substrates of the human sexual behavior are still unknown, however, those same substrates are very well characterized in the rodent female sexual behavior, which has the advantage of being a very simple reflexive response, dependent on the activation of estrogen receptors (ERs) in the ventrolateral division of the hypothalamic ventromedial nucleus (VMNvl) (Pfaff, 1999).

Neurons of VMNvl of the female rat are very responsive to, and dependent on, fluctuating levels of estradiol for the establishment and activation of neuronal connections that will lead to the promotion of the receptive component of the female sexual behavior (revised in Blaustein, 2009). In the absence of estradiol, VMNvl neurons were found to be morphological, biochemical and physiologically quiescent; upon the natural or external raise in estradiol levels, the VMNvl neurons became larger, metabolically active, establish a denser connective network and show increased electrical excitation (revised in Blaustein, 2009; Pfaff, 1999; also see Micevych and Christensen, 2012; Sá and Madeira, 2005). All of these changes culminate in the promotion and establishment of the lordosis reflex (Blaustein, 2009; Micevych and Christensen, 2012; Pfaff, 1999). It was previously demonstrated that the VMNvl expresses both types of ERs, ERα and ERβ, and receive dense projections from brain areas that also express both ERs (Shughrue et al.,

* Corresponding author at: Department of Biomedicine, Unit of Anatomy, Faculty of Medicine, University of Porto, Alameda Hernâni Monteiro, 4200-319, Porto, Portugal.
E-mail address: sasusana@med.up.pt (S.I. Sá).

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1997; Shughrue and Merchenthaler, 2001; Simerly et al., 1990). In the VMNvl, the ERα is abundantly expressed throughout the nucleus, while ERβ-expressing neurons are few and concentrate in its caudal part (Bodo and Rissman, 2006; Shughrue and Merchenthaler, 2001). Earlier studies showed that, in the VMNvl, ERα activation is essential for the expression of progesterone receptor (PR) mRNA and protein, which is crucial for the promotion of the lordosis reflex (Mazzucco et al., 2008; Sá et al., 2009; Walf et al., 2008). Contrariwise, ERβ activation is not able to promote the lordosis reflex or the expression of PRs (Bodo and Rissman, 2006; Mazzucco et al., 2008; Sá et al., 2013), but induces neuronal hypertrophy and synaptogenesis in the VMNvl (Sá et al., 2009). Furthermore, ERβ is involved in other neuroendocrine responses, such as the regulation of gonadotropin-releasing hormone (Bodo and Rissman, 2006; Kalló et al., 2001) and is known for its action in arousal, fear responses, anxiety and learning and memory (Bodo and Rissman, 2006; Lund et al., 2005; Weiser et al., 2008). In a previous study, we have shown that estradiol-responding afferences to the VMNvl have different impact on the nucleus morphology and function (Sá et al., 2010). It was suggested that the VMNvl-dependent behavioral response is dependent on local ERα activation of PR expression, and that the VMNvl neuronal connections enables the perfect synchronization of the behavioral response with the peripheral endocrinology of the female reproductive system, in order to optimize the sexual behavior (Blaustein, 2009; Micevych and Christensen, 2012; Pfaff, 1999). These intrinsic properties of the VMNvl render this hypothalamic nucleus a good model to study the effects of ER interaction in the modulation of the neuronal pathways that translate a neuroendocrine stimulus into a behavioral response, making it relevant in the study of the effects of endocrine disruptors in the promotion of behavioral dysfunctions.

As a selective ER modulator, TAM is able to bind to both ERα and/or ERβ, promoting or inhibiting their action according to different cell and tissue systems (Hall and McDonnell, 1999; Jordan, 2001; Patisaul et al., 2003). In addition, it was has shown that TAM has dissimilar effects according to estradiol levels. In fact, in the absence of estradiol, TAM was shown to have a weak agonistic action through the ERα and an anti-estrogen action through ERβ; contrariwise, in the presence of estradiol, TAM shows antagonistic action through both ERs (Hall and McDonnell, 1999; Kojetin et al., 2008; Martinkovich et al., 2014). It was reported that TAM inhibits the female sexual behavior in estradiol-primed rats, decreases ERα-dependent PR mRNA and protein expression and blocks ERβ anxiolytic actions (Etgen, 1979; Jordan, 2001; Lund et al., 2005; McKenna et al., 1992; Sá et al., 2016).

In this study, our aim is to analyze the implications of systemic TAM administration on the morphological and chemical plasticity of a neuronal population where the dynamics between ERα and ERβ leads to important neuroendocrine responses and try to understand the cellular and molecular basis of TAM-induced sexual dysfunction. In this way, we used an established rat model of induction of behavioral estrus (Flanagan-Cato et al., 2006; Pfaff, 1999; Pfaus et al., 2006) and biochemical and stereological methods to estimate the total number and size of axospinous (spine) and axodendritic (dendritic) synapses established by VMNvl neurons and to determine the changes in the expression of ERα and ERβ in the VMNvl upon TAM administration.

2. Materials and methods

2.1. Animals

Female Wistar rats were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.) and ambient temperature of 23 °C, with food and water continuously available. Throughout the experiments, the estrus cycles were monitored by daily vaginal cytology and only females exhibiting consecutive 4–5 days estrus cycles were ovariectomized and only the ones showing the absence of cyclicity upon ovariectomy were submitted to hormonal therapy. At 10 weeks of age, rats were bilaterally ovariectomized under deep anesthesia by sequential administration of promethazine (10 mg/kg body weight, s.c.), xylazine (2.6 mg/kg body weight, i.m.) and ketamine (50 mg/kg body weight, i.m.). All studies were performed in accordance with the European Communities Council Directives of 22 September 2010 (2010/63/EU) and Portuguese Act n°113/13.

2.2. Treatments

Starting 12 days after ovariectomy, rats were separated in four groups of 14 animals each. The animals were allotted to one of four treatments, upon the administration of two injections (s.c.), 24 h apart, of: 1) 0.1 ml oil (Oil group); 2) 10 μg EB (EB group); 3) 2 mg TAM, each pulse followed 1 h by 10 μg EB (TAM-EB group) or 4) 2 mg TAM (TAM group). EB and TAM were purchased from Sigma-Aldrich Company Ltd. (Madrid, Spain) and were dissolved in 0.1 ml of sesame oil (Sigma-Aldrich Company Ltd., Madrid, Spain). At the end of the experiment, 72 h after the last injection, animals of all groups were anesthetized with 3 ml/kg body weight of a solution containing sodium pentobarbital (10 mg/ml, i.p.) and separated in three different sets of animals. Rats used for structural studies (n = 5 per group) were sacrificed by intracardiac perfusion of a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.2. Rats used for immunohistochemical studies (n = 5 per group) were sacrificed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.6. Animals used for biochemical studies (n = 4 per group) were sacrificed by decapitation. The brains of perfused rats were removed from the skulls, weighed and immersed in the same fixative solution for 1 h, before processing. The brains from decapitated rats were dissected and the right and left VMNvl were isolated and processed together in all biochemical analyses.

2.3. Tissue processing for morphological studies

After post-fixation, the brains were bisected sagitally through the midline. The hemispheres were transected in the coronal plane through the posterior border of the optic chiasm, rostrally, and the posterior limit of the mammillary bodies, caudally. The right and left hemispheres were separated and alternately assigned for processing for electron microscopy or glycolmethacrylate embedding as previously described (Madeira et al., 2001; Sá et al., 2009; Sá and Madeira, 2005). For electron microscopy studies, two blocks of tissue containing the VMNvl of each rat were postfixed with osmium tetroxide (2% in 0.12 M phosphate buffer), dehydrated through graded series of ethanol solutions and stained in 1% uranyl acetate. After passage in propylene oxide, the blocks were embedded in Epon and then re-embedded, according to the isector method (Nyengaard and Gundersen, 1992). From each of the two blocks per rat thus obtained, ten serial 2 μm-thick sections were cut, placed on a gelatin-coated microscope slide and stained with Toluidine blue. Pyramids were trimmed on each block and two or three ribbons of 8–10 serial ultrathin sections were cut, collected on Formvar-coated grids, and double-stained with uranyl acetate and lead citrate.

The blocks of tissue used for glycolmethacrylate-embedding were dehydrated through a series of ethanol solutions before being embedded in glycolmethacrylate (hydroxylethylmethacrylate; Technovit 7100, Kulzerand Co., Wehrheim, Germany) as previously described (Madeira et al., 2001; Sá and Madeira, 2005). The blocks were sectioned in the coronal plane, at 40 μm, using a Jung Multicut microtome. All sections were collected, mounted serially, stained with Giemsa solution and coverslipped with Histomount, as previously described in detail (Madeira et al., 2001; Sá and Madeira, 2005).

2.4. Tissue processing for immunohistochemistry

After post-fixation, the brains were transferred to a solution of 10%