Brain Research Bulletin xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Brain Research Bulletin

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

Research report

Estradiol effect on short-term object memory under hypocholinergic condition

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ARTICLE INFO

Keywords:
Acetylcholine
Estradiol
Object recognition memory
Estrogen receptors

ABSTRACT

Estrogens positively affect object recognition memory (ORM). However, whether this effect rely on acetylcholine is unknown. Here we investigated if 17β-estradiol (E2) would be able to recover ORM deficits in animals with decreased expression of the Vesicular Acetylcholine Transporter (VAChT KD^{HET}). We found that E2 improved short-term ORM (STM) in VAChT KD^{HET} male and in OVX female mutant mice. However, E2 did not recover long-term (LTM) ORM in both sexes. Next, we tested whether hippocampal ERs activation could also rescue STM in mutant mice. Our results showed that ERα seems to be both sufficient and necessary for STM consolidation in female VAChT KD^{HET}. Differently, in male, both ERα and ERβ activation recovered STM. In addition, we tested whether mRNA level of estrogen receptors (ER) is also sensitive to VAChT expression. Female mutant mice showed lower levels of ER alpha (ERα) mRNA in the hippocampus, while no differences in male were observed. Together, our results showed that under hypocholinergic function, E2 improve short-term object recognition in both male and female. Furthermore, we showed that changes in VAChT expression might potentially modulate hippocampal ERα expression in a sex-dependent-manner.

1. Introduction

Hippocampus-dependent memories are strongly affected by estrogens levels. Even a single injection of 17β-estradiol (E2), at the time of memory consolidation, is effective on improving contextual fear (Jasnow et al., 2006), spatial (Daniel et al., 1997; Gibbs, 1999; Gresack and Frick, 2006) and object recognition memories (Boulware et al., 2013; Fernandez et al., 2008; Pereira et al., 2014). Particularly the object recognition memory (ORM) appears to be very sensitive to both acute (Bastos et al., 2015; Boulware et al., 2013; Fernandez et al., 2008; Lewis et al., 2008; Pereira et al., 2014) and chronic E2 effects (Fonseca et al., 2013; Gresack and Frick, 2006).

Estrogens may exert their effects through binding on estrogen receptors (ERα and ERβ) and forming an ER complex in the cytoplasm, with subsequent modulation of gene transcription, effect known as genomic (Nilsson et al., 2001). However, E2 may also act through membrane-bound receptors, activating non-genomic mechanisms (Abraham et al., 2004; Filardo et al., 2000).

The effects of ERs activation on memory are still unclear. DPN, a specific ERβ agonist, can either improve (Boulware et al., 2013; Jacome et al., 2010; Rhodes and Frye, 2006) or have no effect (Pereira et al., 2014; Phan et al., 2011) on ORM. Similarly, ORM can be positively affected (Boulware et al., 2013; Pereira et al., 2014) or unaffected by ERα activation (Jacome et al., 2010; Rhodes and Frye, 2006). Finally, GPER1 activation seems to improve ORM (Gabor et al., 2015; Kim et al., 2016).

On contrary, E2 positive effect on ORM has proved to be very consistent, and its actions are target-dependent. Hippocampal glutamatergic neurons are strongly suggested as the main players mediating the E2 effects on object recognition memory (Boulware et al., 2013; Daniel and Dohanich, 2001; Frick, 2015; Vedder et al., 2013) and hippocampal synaptic plasticity (Smith and McMahon, 2006; Spencer et al., 2008). However, whether E2 would employ other systems than the glutamatergic to improve ORM remains to be fully elucidated.

Cholinergic system has also been suggested as a possible target to E2 actions. Indeed, several studies showed that E2 modulates the expression of cholinergic markers, as well as acetylcholine release (Gibbs, 2000; Gibbs and Pfaff, 1992; Luine, 1985; O'Malley et al., 1987). In the

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https://doi.org/10.1016/j.brainresbull.2018.01.012
Received 24 July 2017; Received in revised form 10 January 2018; Accepted 16 January 2018
0361-9230/ © 2018 Published by Elsevier Inc.

T-maze task, the mnemonic effects of E2 rely on cholinergic system (Farr et al., 2000; Gibbs, 2002, 2007; Gibbs et al., 2011). Furthermore, there are several evidences showing that estrogen-cholinergic interactions may impact on cognitive aging in humans (Newhouse and Dumas, 2015). However, the contribution of the cholinergic system to E2 effects on ORM is unknown.

To test the hypothesis that cholinergic system functionality is important to E2 effects on ORM, we used VAChT KD^{HET} mice. VAChT protein expression is decreased approximately 45% in all tissues of those animals. Therefore, acetylcholine release in the brain of these animals is reduced under basal and evoked conditions (Lima Rde et al., 2010). They also present cognitive deficits in social and object recognition memory (Prado et al., 2006). Interestingly, short-term ORM is preserved in female, but not in male mutant mice, while LTM is impaired in both sexes (Capettini et al., 2011). Here we evaluated the effect of E2, as well as ERs agonists, in the ORM of both male and female VAChT KD^{HET} mice. Furthermore, we investigated if VAChT expression would impact on ERs mRNA levels in the hippocampus and basal forebrain.

2. Material and methods

2.1. Animals

Adult (12–16 weeks old) male and female VAChT KD^{HET} mice were used (Prado et al., 2006). All control animals were wild-type (WT) littermates of the same generation and genetic background. Mice were housed in groups of three to five per cage in a temperature-controlled room (22 ± 2C) with a 12:12 light–dark cycles. Food and water were provided *ad libitum*. All experimental procedures were carried out in the light phase and approved by the Institutional Animal Care and Use Committees of Universidade Federal de Minas Gerais (228/2013). All experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Novel object recognition task

Novel object recognition task assesses a non-spatial hippocampal dependent memory (Baker and Kim, 2002; Clark et al., 2000). The protocol applied was described previously (Capettini et al., 2011). Briefly, in the first day, mice were placed in an empty black box (50 cm × 40 cm × 20 cm) equally illuminated and allowed to freely explore it for 20 min. Twenty-four hours later, in the sample phase; mice were re-habituated to the empty box for 1 min. Then, two identical objects were placed in the left and right side of the box (~7 cm from the wall) and mice were allowed to investigate the objects for 10 min. In the choice phase, a novel object replaced one of the familiar objects and mice were allowed to investigate the objects for another 10 min. To assess short-term (STM) and long-term (LTM) memories, mice were submitted to the choice phase 1h30 and 24 h after the sample phase, respectively. Time spent with each object was recorded. Results are expressed as recognition index [time exploring the new object/total time of exploration]. The placement of novel objects was counterbalanced across mice.

2.3. Ovariectomy

Animals were anesthetized with isoflurane (induction up to 4%, 1–3% for maintenance). An incision was made just lateral to the midline at the pelvic level. The ovary, oviduct and top of the fallopian tubes were bilaterally clamped and removed (Capettini et al., 2011). In the SHAM group the procedure was similar but the ovaries were only exposed and not removed. The abdominal wall and the skin were sutured. At the end of the surgery, they received a single intra-muscular injection of Flunixin meglumine (Banamine; 0.3 mg/kg) and pentabiotic (160 mg/kg; Fort Dodge Animal Health, Brazil) and recovered for at least 5 days.

2.4. Stereotaxic surgery

Animals were anesthetized with isoflurane (induction up to 4%, 1–3% for maintenance) and placed in a stereotaxic apparatus. Bregma and lambda were aligned at the same horizontal and vertical planes. Small holes (~0.7 mm) were drilled directed toward the CA1 region of dorsal hippocampus (from bregma: AP: −1.9; LL: ± 1.6; DV: −1.0) (Paxinos and Franklin, 2001) and bilateral guide cannulae (22G, 7 mm) with inserted dummy cannulae were fixed in the skull with zinc cement followed by dental acrylic (Pereira et al., 2014). At the end of the surgery, they received a single intra-muscular injection of Flunixin meglumine (Banamine; 0.3 mg/kg) and pentabiotic (160 mg/kg; Fort Dodge Animal Health, Brazil) and recovered for at least 5 days. Ovariectomized animals that also had cannulae implantation into the hippocampus were submitted to both surgeries at the same time.

2.5. Drugs and infusions

All drugs were administered immediately after the sample phase of object recognition task. Mice were gently held and the dummy cannulae were removed. An injector cannula (30G, 8 mm) was coupled to the guide cannula at the time of the infusions. A microinfusion pump was used to control infusions through polyethylene tubing (PE20) connected to a 10 µl syringe (Hamilton). Drugs were infused bilaterally into the CA1 region of the dorsal hippocampus at a rate of 0.5 µl/min in a volume of 0.5 µl/side. Injector cannula remained in place for 1 min after infusion to avoid drug diffusion into injection track.

Hydroxy-betacyclodextrin-encapsulated (HβC) E2 (Sigma–Aldrich) or vehicle (HβC) was administered intraperitoneally (i.p.) at 0.2 mg/kg or intra-hippocampal (i.h.) at 10 µg/µl. We chose those doses because they both improved 48 h object memory in C57/BL6 female mice (Fernandez et al., 2008). ERα agonist, (4,40,400-[4-propyl-(1H)-pyrazole-1,3,5-triyl]-tris-phenol [PPT Sigma–Aldrich]) has a 410-fold greater affinity for ERα over ERβ (Stauffer et al., 2000). PPT was dissolved in 50% DMSO and was administered i.h. at 1 µg/µl (Pereira et al., 2014). ERβ agonist, (2,3-bis(4-hydroxyphenyl)-propionitrile [DPN Sigma–Aldrich]) has a 70-fold greater affinity for ERβ over ERα (Meyers et al., 2001). DPN was dissolved in 50% DMSO and administered i.h. at 2 µg/µl (Walf and Frye, 2007). Those doses were used in previous studies from our group and had the same effect of lower doses (Pereira et. al., 2014). ERα selective antagonist (theophylline, 8 [(benzylthio) methyl]-(7CI,8CI) [TPBM Sigma–Aldrich]) was dissolved in 20% DMSO and was administered i.h. at 250 ng/µl (Mao et al., 2008). In order to ensure that the observed effects were due to cholinergic hypofunction animals were treated with galantamine, an acetylcholinesterase inhibitor. Galantamine was dissolved in saline and was administered i.p. at 1 mg/Kg (Prado et al., 2006).

2.6. Histology

At the end of the experiments, mice were euthanized and their brains immediately removed and stored in 4% paraformaldehyde for one day, followed by two days in 30% sucrose. Coronal sections (100 µm thick collected proximal to cannulae tracts) were cut on a cryostat (–20 °C). Slides were stained with neutral red and injection sites were verified by light microscope. Only mice with correct cannulae placements were included in statistical analyses (Pereira et al., 2014).

2.7. Reverse transcription quantitative polymerase chain reaction

Naïve animals were euthanized and their brains were rapidly removed. Hippocampus and basal forebrain were bilaterally dissected on

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