Effects of estrogen and progesterone treatment on rat hippocampal NMDA receptors: Relationship to Morris water maze performance

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Received: June 23, 2004; Accepted: November 2, 2004

Abstract

Estrogen modulates NMDA receptors function in the brain. It increases both dendritic spine density and synapse number in the hippocampus, an effect that can be blocked by NMDA antagonist. In this study, we investigated the effect of 17 β -estradiol and progesterone treatment on NMDA receptors in ovariectomized rats. Two different doses were used for 10 weeks. Receptor autoradiography was done on brain sections using [³H] MK-801 as a ligand. Our results showed a significant increase in [³H] MK-801 binding in the dentate gyrus, CA3 and CA4 areas of the hippocampus of ovariectomized compared to sham operated rats. In addition, we observed similar changes in CA1. 17 β -estradiol treatment in both doses reduced the binding back to the normal level while progesterone treatment did not show any effect. Spatial reference memory was tested on Morris water maze task. Ovariectomy severely impaired spatial reference memory. Estradiol but not progesterone treatment significantly improved the memory performance of the ovariectomized rats. Low dose treatment showed better learning than high dose estrogen treatment. The decrease in the antagonist sites by estradiol treatment could result in an increase in the sensitivity of the hippocampus to the excitatory stimulation by glutamate system and hence the effect of estradiol on learning and memory. The changes of NMDA receptors in the hippocampus support the concept that estrogen-enhancing effect on spatial reference memory could be through the enhancing of NMDA function.

Keywords: ovariectomy • estradiol • progesterone • reference memory • MK-801 • NMDA

Introduction

Ovarian hormones have several effects on the nervous system that extend beyond regulation of the reproductive functions. Estrogen is believed to have

a neuroprotective effect, both in human [1] and animal models [2]. Estrogen protects the neurons from injury of different insults like β -amyloid and glutamate toxicity [3], oxidative stress [4] and ischemia [5]. Moreover, it enhances neuronal repair after injury [6] and neurite plasticity by regulating the number of synaptic inputs in pyramidal cells of the CA1 region in the hippocampus [7, 8]. It has been

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shown earlier that estrogen increased the sensitivity of the hippocampal CA1 pyramidal cells to NMDA receptor mediated synaptic input [9]. Estradiol increases NMDA agonist binding as well as the NR1 subunit level in CA1 dendrites [10, 11]. Moreover, estradiol-induced increase of the dendritic spine density is blocked by NMDA receptors antagonists.

NMDA receptors are thought to be involved in estrogen mediated neuroprotective effect. Being expressed in abundance in the brain regions involved in memory, like the hippocampus and cortex, NMDA receptors play a vital role in learning and memory [12, 13]. They are essential for the induction of plasticity (long-term potentiation) in the CA1 field and dentate gyrus [14]. L-glutamate, the major excitatory amino acid neurotransmitter in the brain, is involved in many neurologic functions such as cognition and memory [15]. It activates three major classes of receptors, the NMDA (N-Methyl-D-Aspartate) ionotropic receptors, the non NMDA kainate/AMPA (α -amino-3-hydroxy-5-methyl-4-isoxasole propionic acid) ionotropic receptors, and the glutamate metabotropic receptors [16]. However, it has been proposed that the disruption of glutamate metabolism and glutamate receptor-mediated neurotoxicity, neurotoxicity also referred to as excitotoxicity [17], represents one of the major mechanisms of neuronal death in many neurodegenerative disorders such as Alzheimer's disease AD, as well as in other pathological conditions such as ischemia and epilepsy [18, 19]. Improvement of memory performance after estrogen replacement therapy in postmenopausal women, as well as memory fluctuation with estrogen levels during the menstrual cycle was documented [20]. Moreover, clinical evidences showed that estrogen exposure decreased the risk and delayed the onset and progress of AD [21]. Recent studies suggest that estrogen replacement therapy in women can help to prevent or delay the development of AD and reduce the memory decline observed during normal aging and menopause [22-24]. Pronounced effects of estrogen on spatial working memory in different paradigms in ovariectomized rats treated with estrogen was published earlier [2, 25-28]. Conversely, estrogen has no effect on reference memory and it has been shown earlier that it impairs reference memory in Morris water maze task when given in high doses [29].

In order to explore the relationship between ovariectomy and hormone treatment, spatial memory

and hippocampal NMDA glutamate system, we investigated the binding of MK-801 as well as spatial reference memory performance in ovariectomized and ovarian hormones treated rats.

Materials and methods

Animals

In this study female Sprague-Dawley rats (Six/seven month-old, mean body weight 359 ± 13 g) obtained from Charles River (Uppsala, Sweden) were used. They were maintained on a 12-h on, 12-h off lighting schedule with food and water available ad libitum. The rats were kept in single cages. All handling and the study protocol were conducted after the approval by the local ethical committee.

Surgery and anesthesia

After a two-week adaptation period, rats were randomly divided into ovariectomized (OVX) ($n=35$), sham-operated (S) ($n=7$). Ovariectomy was performed through flank incision under sodium pentobarbital anesthesia (60mg/kg i.p.). Similar surgical procedures were applied to the Sham group except that the ovaries were not removed. All the efforts were made to minimize both the suffering and the number of animals used.

Treatment

After surgery the ovariectomized rats were then divided into five equal groups and given treatment of either 17β -estradiol (OVX+E2), progesterone (OVX+P) or the vehicle only (OVX+V). The injections were started 7 days after surgery and were given subcutaneously at a dose of either 40 μ g/kg (HE2) or 4 μ g/kg (LE2) for estrogen and 0.5 mg/kg (HP) or 0.05 mg/kg (LP) for progesterone; an equal volume of the vehicle (V) was given to the vehicle group. The injections were given every four days. Treatment continued for 10 weeks. The hormones were prepared every five weeks by dissolving 17β -estradiol (E2) (Sigma, Stockholm, Sweden) or progesterone in corn oil base containing 5% ethanol. The preparation was kept, away from light, at room temperature. At the end of the experiment, the rats were all sacrificed by decapitation under sodium pentobarbital (60mg/kg i.p.) anesthesia. After sacrifice, the brains were removed and immediately frozen in isopentane pre-cooled in liquid nitrogen (-150°C), and stored at -70°C until used for experiment.

Fig.1 Effect of long term ovariectomy and ovarian hormone treatment on spatial learning in the rat. Mean $\pm$ SEM (n= 7) values are shown as the time required to find an invisible platform submerged in water (escape latency). (**p<0.0001) vs SHAM (S) and (##p<0.005) and (###p<0.0005) vs Vehicle (V).

Morris water maze test

The water maze was modified from Morris [30]. Animals were trained in a pool 1.40 m diameter and 0.3 m high, containing water held constant at $22 \pm 1^\circ\text{C}$. The pool was in the centre of a room containing various salient visual cues. A 10x10 cm transparent platform was hidden in a constant place in the pool with its top surface submerged 1.5 cm below the water level. Training consisted of four trials per day for 4 days. One day after the last training session, the rats were given one 60 s retention test trial in which platform was removed from the tank.

Receptor autoradiography

Coronal brain sections, 10 μm thick were cut at the level of the habenular commissures of the dorsal hippocampus at -20°C . NMDA receptor autoradiography was performed as described previously [31]. Briefly, the sections were pre-washed at room temperature in 50mM $\text{Na}^+\text{-K}^+$ phosphate buffer (pH 7.4) 15 min. The sections were then incubated in Tris-HCl buffer containing 1 μM glutamate, 100 μM glycine, 1mM EDTA, 0.43mM MgCl_2 and 2nM [^3H] MK-801 (sp. Ac. 22.5Ci/mmol; NEN Life Science products) at 25°C for 120 min. MK-801 (100 μM) was used to determine non-specific binding. At the end of the incubation period the sections

were rinsed in the buffer to terminate the assay and dipped in distilled water to remove excess buffer salts. Subsequently, the sections were dried under a stream of cold air.

Autoradiograms were obtained by exposing the labeled sections to tritium sensitive films (HyperfilmTM- ^3H , Amersham, UK) in X-ray cassettes for 1 month period at -20°C . Calibrated radioactive standards (Amersham) were exposed and developed in parallel with tissue sections to permit conversion of mean grey densities to molar concentrations of receptor-bound radio-ligand. Quantitative analysis of the autoradiograms was performed using computer-assisted image analysis (NIH image). Specific binding was obtained by subtracting the non-specific binding from the total binding. The specific binding was expressed in fmol/mg tissue wet weight.

Statistics

Statistical analysis was performed using analysis of variance (ANOVA) repeated measures for comparing the cognitive performance in Morris water maze between experimental groups and one way analysis of variance (ANOVA) for the autoradiograms. Post hoc Fisher's PLSD, were applied for evaluation of significant differences between the groups. Significance was considered at ($p < 0.05$). Data were expressed in mean ($\pm$ SEM).

Fig.2 Retention test of the Morris water maze task are shown as group mean $\pm$ S.E.M. values of the total time (during a 60s probe trial) the animal spent in the quadrant where the platform was originally located. (# $p<0.05$) and (## $p<0.005$) vs Vehicle (V), and (* $p<0.05$) vs Sham (S).

Results

Morris water maze

In the Morris water maze a significant difference was found between ovariectomized vehicle treated ($p<0.0001$) and sham operated group on escape distance and platform finding on the 3rd and 4th days of the trial. The estradiol treatment in both doses showed better acquisition than the vehicle treatment. Significantly better learning compared to ovariec-

tomized rats was shown by LE2 rats ($p<0.005$) all through the test days, while HE2 ($p<0.0005$) on the 3rd and 4th days of the experiment. Progesterone treated groups did not show significant learning compared to the vehicle treated ones (Fig. 1). In the retention test compared with ovariectomized and progesterone treated rats, estrogen treated rats spent longer time in the quadrant where the platform was previously located. Moreover, significance was shown by HE2 rats ($p<0.005$) and LE2 ($p<0.05$) compared to the vehicle group (Fig. 2). There was no

Fig.3 Photomicrograph showing the autoradiograms of [³H] MK-801 binding in; (a) Sham-operated control, (b) Vehicle, (c) HE2, (d) HP and (e) non-specific binding.

speed difference among the different groups (data not shown).

Receptor Autoradiography

In this study receptor autoradiography was used to compare the distribution of [³H] MK-801 binding density in hippocampus of sham operated rats as well as ovariectomized rats with vehicle, estradiol or progesterone treatment. Receptor quantification was performed in the hippocampus in CA1, CA3, CA4 and dentate gyrus. Binding of NMDA was highest in the stratum radiatum and the stratum oriens of hippocampus and in the molecular layer of the dentate gyrus compared to that of the neuronal layer and pyramidal layers respectively in the entire groups studied. Lower level of binding was observed in the CA3 area compared to the CA1 and CA4 (Fig. 3). Our results confirm previous observations related to NMDA receptor binding pattern [32].

In addition our results show a significant increase in [³H] MK-801 binding in the dentate gyrus ($p<0.005$), CA1 ($p<0.005$), CA3 ($p<0.05$) and CA4 ($p<0.05$) (Fig. 4A, B, C, D) areas of the hippocampus of ovariectomized rats when compared with sham control rats.

Estrogen treatment reduced the [³H] MK-801 binding density back to the sham level. When compared with the ovariectomized vehicle treated rats HE2 and LE2 treatment significantly reduced the [³H] MK-801 binding levels in the dentate gyrus ($p<0.05$), CA1 ($p<0.005$), CA3 ($p<0.05$) and CA4 ($p<0.01$) (Fig. 3A, B, C, D). No significant difference between the two estradiol doses used in our study was noticed.

Progesterone treatment did not significantly affect [³H] MK-801 binding density.

Discussion

In this study we investigated the effect of ovariectomy and ovarian hormones treatment on hippocampal NMDA receptors in order to understand the interaction of estradiol and the glutamate system. MK-801 was used as a ligand, which is a non-competitive NMDA receptor antagonist, binds to block the channel in its open state [33]. Our results showed that a

significant increase in MK-801 binding density in the hippocampus and dentate gyrus in the ovariectomized rats compared to the control. Following estradiol treatment MK-801 binding density was restored back to the control level in all areas of the hippocampus especially in the high dose treatment. In contrast, progesterone has no effect on the ovariectomy induced changes in these areas. Our results are in agreement with those of Weiland [10], which showed that estradiol significantly decreased the MK-801 binding in the dentate gyrus, CA3 and CA4 of ovariectomized rats. However, in addition to Weiland's results, we see a significant decrease in MK-801 binding in the CA1 area after estradiol treatment. NMDA activity in CA1 is important for induction of long-term potentiation and the storage of information. Decrease of MK-801 binding seen in our result implies therefore that estrogen's positive effect upon memory may be through the enhancement of NMDA in CA1 region of the hippocampus function. The decrease in the antagonist sites by estradiol treatment could result in an increase in the sensitivity of the hippocampus to the excitatory stimulation by glutamate system and hence the effect of estradiol on learning and memory. Estradiol has a well known effect on the CA1 area. Treatment with estradiol after ovariectomy increases the dendritic spine density and synapse in CA1 pyramidal cells [7, 34]. Estradiol effects on the dendritic spine density was found to be blocked by NMDA receptors antagonists and not AMPA or other neurotransmitters receptors antagonists [35], confirming that the NMDA receptors are central key players in the estradiol induced increase in dendritic spine density.

Our results in Morris water maze task showed a significant difference in spatial learning behavior between ovariectomized vehicle treated and sham operated rats. Improvement in learning was achieved after estradiol treatment. Our result is in agreement with previous result that proved the positive effect of estradiol on spatial reference memory in aged mice [36]. Both doses used in our study, increased acquisition of spatial learning and retention, but low dose treatment group showed better learning than the high dose estradiol treatment. Estradiol enhancement of acquisition of spatial working memory was reported earlier even with different paradigms of memory measurement [26]. Ovariectomy and its effect on the spatial reference memory have been somewhat conflicting. While

Fig. 4 Bar graphs showing the effect of ovariectomy on [³H] MK-801 binding density in; (A) Dentate gyrus (B) CA1, (C) CA3, and (D) CA4. Data are expressed as mean ($\pm$ S.E.M) (n= 5-7). (**p<0.005) vs SHAM (S) and (##p<0.005) and (###p<0.005) vs Vehicle (V).

some studies report no effect of ovariectomy on spatial reference memory [37], yet others found reversible memory impairment by ovariectomy [2]. Similarly controversial results were obtained by estradiol treatment on spatial reference memory. While estradiol reverse the ovariectomy induced memory impairment in some studies [2], it improved only working memory and has no effect on spatial reference memory in other studies [25].

The discrepancy in the results can be due to variations in the experimental conditions between the different studies, e.g. use of different tasks, age of the rats, doses and regimens of treatment. It is believed that the memory enhancing effect of estrogen involves interaction of estrogen with different neurotransmitters system. Estradiol interaction with the brain cholinergic system has been shown earlier [38, 39]. Moreover, estradiol affects the monoaminergic

neurotransmitters and excitatory neurotransmitters [40]. It was reported that estradiol regulates the agonist and antagonist binding sites on the NMDA receptors/ion channel complex independently. Estradiol increased the NMDA agonist and competitive inhibitor binding and decreased the MK-801 binding sites [10]. Estradiol induced regulation of spatial memory and NMDA receptors is likely to be mediated by the two nuclear estrogen receptors, estrogen receptor α (ER α) and β (ER β) [41]. Both receptors are expressed in the hippocampus and neocortex [42]. There is also the possibility of indirect effect through estrogen interaction with other neurotransmitter systems which in turn affect the glutamate system. It has earlier been shown that the ability of estrogen to alter NMDA receptor binding in CA1 is related to its ability to alter cholinergic system [43].

Taken together our results and previous results show that estradiol plays a dual effect on NMDA receptors. It enhances the cognitive function and at the same time exert a neuroprotective effect [44]. Estrogen is thought to be responsible for memory fluctuation during the menstrual cycle [20]. Estrogen replacement therapy improved memory performance in postmenopausal women. Moreover, clinical evidences showed that estrogen exposure decreased the risk and delayed the onset and progress of Alzheimer's disease [21]. Recent studies suggest that estrogen replacement therapy in women can help to prevent or delay the development of Alzheimer's disease and reduce the memory decline observed during normal aging and menopause [22-24].

In conclusion, our results showed that estradiol improves spatial reference memory, more pronounced by low dose estrogen treatment in ovariectomized rats. Moreover, we see MK-801 binding changes in CA1 area which was not reported before. NMDA receptor functions in the hippocampus do seem to be regulated by estradiol. These findings provide insights of the interaction of estradiol and the glutamate system especially in relation to postmenopausal memory problems.

Acknowledgements

This study was supported by research grants from the Swedish Medical Research Council and The Gamla Tjänarinnor stiftelsen.

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