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Molecular Brain Research 140 (2005) 127 – 132
MOLECULAR BRAIN RESEARCH
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Short Communication

Hippocampal NMDA receptor subunit expression and watermaze learning in estrogen deficient female mice

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Accepted 9 July 2005
Available online 9 August 2005

Abstract

The aromatase knockout (ArKO) mouse is estrogen deficient. Using reverse-transcription and real-time PCR, we showed that transcript levels of the *N*-methyl-D-aspartate (NMDA) receptor subunit NR2B are significantly higher in the hippocampus of female ArKO mice compared to wild-type (WT) littermates. Expression levels of NR1, NR2A, but not NR2C, also tended to be higher in ArKO mice. In the Morris watermaze test for spatial memory, both genotypes displayed equal significant improvement in the latency in locating the invisible platform over the 5-day training period. These findings show that selective loss of estrogen synthesis is associated with changes in NMDA receptor subunit expression in the hippocampus but little change in spatial learning ability.

Theme: Neurotransmitters, modulators, transporters and receptors
Topic: Excitatory amino acid receptors: physiology, pharmacology and modulation

Keywords: Aromatase knockout; ArKO; Female; Estrogen; Hippocampus; Long-term spatial memory; *N*-methyl-D-aspartate (NMDA) receptor subunit; Morris watermaze

Epidemiologic evidence suggests that estrogen replacement therapy may decrease the incidence of Alzheimer's and Parkinson's diseases by delaying their onset and slowing the decline in cognitive functions associated with these neurodegenerative diseases [6,25]. One possibility is that estrogen is having an anti-oxidant effect as neurons of Alzheimer's patients suffer from oxidative stress. Estrogens may also promote the growth of cholinergic neurones and reduce β-amyloid degeneration, the recognisable feature of Alzheimer's disease [1]. On the other hand, the data from the Women's Health Initiative Memory Study suggested an increased risk for all types of dementia in postmenopausal women taking combined conjugated equine estrogens plus progestin [22,26]. Hence, there is still controversy on the effects of estrogens on cognition.

N-methyl-D-aspartate (NMDA) receptors are crucial for normal CNS function and are ubiquitously distributed in high levels throughout the brain. NMDA receptors are heteromeric complexes of different subunits. There are two types of subunits known, NR1 and NR2. The latter consists of 4 subtypes: NR2A, NR2B, NR2C and NR2D. Each NMDA receptor consists of one NR1 subunit and at least one NR2 subunit (for review, see [15]). The identity of the NR2 subunit determines the molecular composition and functional properties of the receptor and hence, NMDA receptor channel diversity [15]. The expression in the brain of the different NMDA receptor subunits varies between brain regions and also during life [31].

Abbreviations: ArKO, aromatase knockout; WT, wild-type; NMDA, *N*-methyl-D-aspartate; NR, *N*-methyl-D-aspartate receptor; NR1, *N*-methyl-D-aspartate receptor subunit 1; NR2, *N*-methyl-D-aspartate receptor subunit 2

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doi:10.1016/j.molbrainres.2005.07.004

NMDA receptors in the hippocampus are involved in long-term potentiation (LTP) [15]. Furthermore, several studies have suggested an important role of NMDA receptors in learning and memory [2]. This role is under the influence of estrogen and other sex steroid hormones [20]. In addition, estrogen is involved in the formation of excitatory NMDA synapses in the hippocampus [17]. To further study the interaction of estrogen, NMDA receptor expression and learning and memory, our aims were to compare the transcript levels of the NMDA subunits (NR1 and NR2A-C) in the hippocampus of the estrogen deficient Aromatase Knockout (ArKO) mouse to those of wild-type (WT) control mice. In addition, we compared performance of these mice in the Morris watermaze test for long-term spatial memory which is dependent on the hippocampus [19]. The ArKO is estrogen deficient because it lacks a functional aromatase, the enzyme that catalyses the conversion of androgens to estrogens [8].

ArKO mice (129SV/J X C57BL/6J) were generated by disruption of the *Cyp19* gene by homologous recombination [8]. Homologous null or WT offspring was bred by crossing mice heterozygous for the disrupted gene. The pups were genotyped by polymerase chain reaction (PCR) as described previously [23]. Animals were housed under SPF conditions and had ad libitum access to water and soy-free mouse chow (Glen Forrest Stockfeeders, WA, Australia). All procedures and experiments were done along the guidelines of the Code of Practice for the Care and Use of Animals for Scientific Research and were approved by the relevant Animal Experimentation Ethics Committees.

For the gene expression studies, female mice of 10–12 weeks old (n = 5) were killed by cervical dislocation and brains were removed and immersed in RNAlater™ (Ambion Inc, TX, USA). Whole hippocampi were dissected out and total RNA was isolated from tissue using the Ultraspec RNA Isolation System (Biotecx, Texas USA). Any DNA contamination was removed by treatment with DNase I (DNA-free kit, Ambion Inc, TX, USA). First strand cDNA was reverse transcribed from 1 μg of total RNA with AMV-reverse transcriptase (Roche, Mannheim, Germany) and random hexamers (GibCo BRL, Paisley, UK) at 42 °C, 30 min followed by 55 °C, 15 min. Real-time PCR was performed on the cDNA using FastStart DNA Master SYBR Green I Kit (Roche, Mannheim, Germany) and specific PCR primers (Table 1; GeneWorks Pty., Ltd., VIC, Australia) in the Roche LightCycler (Roche, Mannheim, Germany). Transcript levels of NR1, NR2A, NR2B and NR2C, were determined by comparison to standard curves. Standards were cDNAs amplified from WT mouse hippocampus total RNA by RT-PCR and checked by sequence analysis. We failed to detect any level of NR2D in the hippocampus. Cyclophilin was used as a housekeeping gene. This was chosen as it is known not to be influenced by estrogen in the brain [32]. The real-time PCR result of each NMDA receptor subunit was normalised against cyclophilin data. Differences between groups were analysed using one way ANOVA (SPSS Inc, USA).

In the Morris watermaze test, female mice of 14–16 weeks old (n = 9 for wildtypes, n = 7 for ArKO mice) were placed in a wide circular arena (150 cm diameter) filled with water made opaque with a small amount of non-allergenic, water soluble white paint. The mice were required to learn the location of an escape platform hidden 1 cm under the surface. The main protocol consisted of 5 consecutive learning days, each with 5 invisible platform trials of maximally 1 min. In addition, on the beginning of day 1, end of day 4, end of day 5, beginning of day 6 and end of day 6, a free-swim trial was conducted (2 min) to assess preference for the quadrant in which the invisible platform is normally found. On day 6, five visible platform trials were conducted. Behaviour of the mice was recorded on video and analysed using the Ethovision video tracking system (Noldus, Wageningen, The Netherlands). Measurements taken were time to reach the platform (latency, s), distance moved to reach the platform (cm), and velocity of movements (cm/s). Place preference in the free-swim trials was calculated as percentage of time spent in the platform quadrant. Data were expressed as mean ± standard error of the mean (SEM) analysed using two-way ANOVA with repeated measures where appropriate (Systat 9, SPSS, USA). Factors were genotype and experimental session.

Expression levels of NR1 showed a trend towards an increase in the female ArKO hippocampi when compared to WT counterparts (P = 0.080; Fig. 1A). Similarly, transcript

Table 1
Primers for real-time PCR

Transcript	Primers (5′ → 3′)	Annealing temp (°C)	Product size (bp)
NR1	forward CAG GAG CGG GTA AAC AAC AGC AAC reverse GAC AGC CCC ACC AGC AGC CAC AGT	58	290
NR2A	forward AGC CCC CTT CGT CAT CGT AGA reverse CAG AAG GGG AAA CAG TGC CAT TA	60	400
NR2B	forward TCC GCC GTG AGT CTT CTG TCT ATG reverse CTG GGT GGT AAA GGG TGG GTT GTC	58	300
NR2C	forward GAT GCC GCC GTC CTC AAC TAC A reverse GCT CCC AGG CAA AGA CCA GAA GG	60	320
Cyclophilin	forward CTT GGG CCG CGT CTC CTT C reverse TGC CGC CAG TGC CAT TAT	60	179

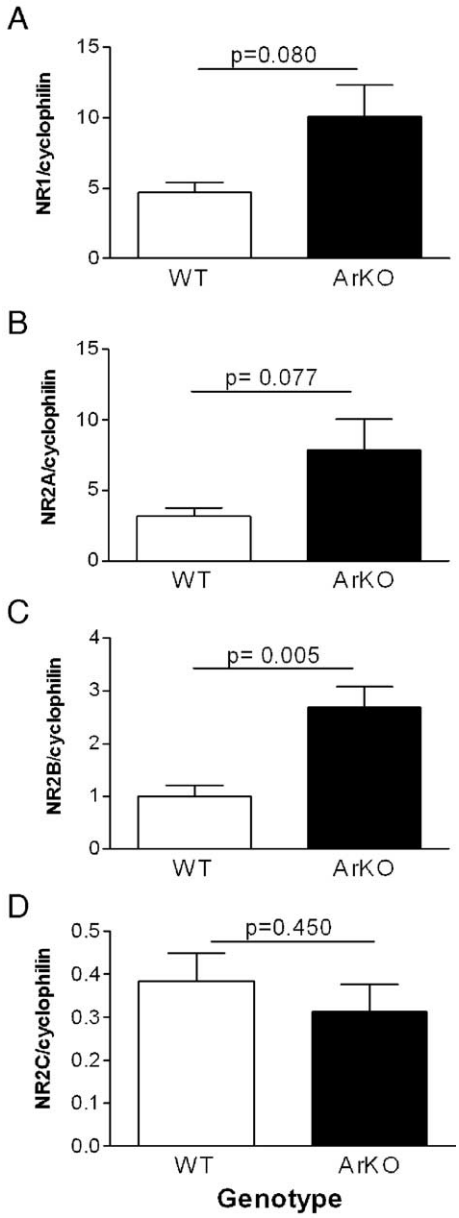

Fig. 1. Hippocampal NR1 (A), NR2A (B), NR2B (C) and NR2C (D) transcript levels in female ArKO and WT mice. Transcript levels were quantitated by reverse transcription of total RNA followed by real-time PCR and normalised to cyclophilin transcript levels; $n = 5$ per group. Data = mean ± SEM.

levels of NR2A showed a non-significant increase in the ArKO mice ($P = 0.077$; Fig. 1B). In contrast, there was a highly significant increase in the expression level of NR2B in the ArKO as compared to WT littermates ($P = 0.005$; Fig. 1C). There was no significant difference in the expression of NR2C between groups ($P = 0.45$; Fig. 1D).

In the Morris watermaze test, both genotypes showed a modest but significant reduction of the latency to find the hidden platform over the 5 testing days (Fig. 2A). This could be interpreted as spatial learning, an ability apparently similar in female WT and ArKO mice. There was also no change between genotypes in distance moved or swim velocity (data not shown). For example, distance moved on day 1 was 633 ± 60 cm vs. 609 ± 40 cm in wildtype controls and ArKO mice, respectively, whereas on day 5 it was 566 ± 24 vs. 531 ± 71 cm. Swim velocity on these days was 11.4 ± 1.0 vs. 11.6 ± 0.8 cm/s and 14.9 ± 0.9 vs. 15.0 ± 1.1 cm/s. Some subtle evidence of differences in learning ability was found when the free-swim trials were analysed (Fig. 2B). ArKO mice showed significant improvement ($P < 0.05$) between the five free-swim trials (Fig. 2B). They spent a significantly higher percentage of time in the quadrant (Fig. 2B) where the invisible platform would normally be found during free-swim trial number 4 (i.e. after 5 days of training) as compared to that during free-swim trial number 1 (i.e. before any training). The last free-swim trial (i.e. trial 5), after the visible platform trial, shows the expected sharp decline in preference. Interestingly, none of these changes were found in WT mice. The visible platform trials showed no difference between the genotypes (wildtypes 15.7 ± 5.8 s

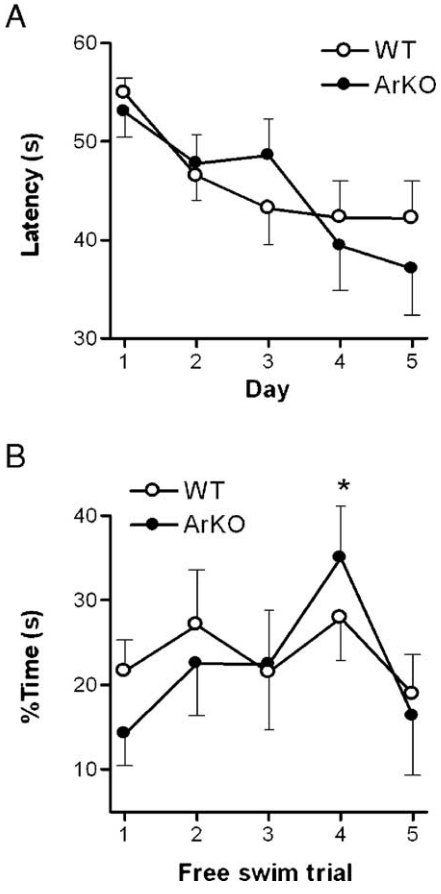

Fig. 2. Results of Morris watermaze test of 14- to 16-week-old female ArKO ($n = 7$) and WT mice ($n = 9$). (A) Time taken for mice to find the hidden platform during the 5-day training. There was an overall effect of Day ($F(4,56) = 7.4$, $P < 0.001$) but no difference between genotypes. (B) Percentage of time spent in the hidden platform quadrant during the free-swim trial. Over the five free-swim trials, there were significant overall differences ($F(4,56) = 3.6$, $P = 0.012$). Separate ANOVA showed significant differences between trials in the ArKO mice ($F(4,24) = 3.4$, $P = 0.024$) but not WT controls. *$P < 0.05$ for difference with percentage time during trial 1 in ArKO but not WT mice.

vs. ArKO 17.2 ± 3.4 s), suggesting no differences in swimming capabilities.

The activation of NMDA receptors has been implicated in long-term memory formation and learning behaviour [2,15,20]. By using reverse transcription and real-time PCR analysis, we demonstrated that the transcript levels of the NMDA receptor 2B subunit are increased significantly in the hippocampi of 10- to 12 week-old female ArKO mice. In addition, the transcripts of NR1 and NR2A subunits showed a trend to increased levels. It is possible, that with a greater number of animals per group, these differences would have become significant. However, these mice showed normal watermaze learning, as shown by equal fall in latency times to reach the hidden platform. Only in one of the free-swim trials was there a tendency for ArKO mice to perform slightly better than controls.

NR1 is the key subunit present in every NMDA receptor channel. There are 8 splice-variants known in 3 exons in a total of 22 exons. This subunit consists of the glycine-binding site and contributes to the Ca^{2+} permeability of the NMDA receptor channel [34]. The NR2A contains the glutamate-binding site and influences the threshold of LTP [13]. In addition, the NR2A subunit is involved in the activation of the calcium/calmodulin kinase II alpha/beta (CaMKIIβ and CAMKIIα) pathway [18], which plays an important role in memory storage. NR2A also plays a role in synaptic plasticity [24], therefore, it may be important for some forms of learning. Using NR2A and NR2C knockout mouse models, it has been demonstrated that combined loss of the NR2A and the NR2C results in impairment of motor co-ordination, whereas a deficiency of only one of the subunits does not have any effect suggesting they can complement each other [12]. NR2C is mainly found in the cerebellum [31]. When compared to the NR1–NR2A or NR1–NR2B combination, a NR1–NR2C combination produced a lower conductance channel [28]. The NR2B subunit was shown in to be essential for both neuronal pattern formation and synaptic plasticity [14,27]. In addition, NR2B is critical in plasticity and memory formation, as shown by improved learning and memory ability in mice overexpressing this subunit [29].

The increase in the hippocampus of ArKO mice in the expression of NR2B and, to a lesser extent NR2A and NR1, would be expected to have several effects on learning and memory. Only NR2C has not been shown to be involved in LTP or learning or memory processes and, in the present study, this was the only subunit that did not show any difference in the transcript level between the genotypes. However, the results of the Morris watermaze test showed that the spatial learning behaviour of female ArKO mice, expressed as escape latency or distance moved, was not altered. The only effect that was seen was slightly better spatial memory compared to WT mice in the free-swim trials.

Aromatase and estrogen receptors have been localised in the hippocampus [11,21]. Therefore, it is reasonable to speculate that estrogen would have effects on the expression levels of NMDA receptor subunits to influence memory processes. From our data, it seems that the effect on NMDA receptor expression is inhibitory as absence of estrogen production in ArKO mice enhances the expression of NMDA receptor subunits (NR1, NR2A and NR2B) which are involved in learning and memory processes. However, a clear link between these changes in levels of NMDA receptor subunits and learning and memory in these mice was not seen. The absence of changes in watermaze performance, as assessed by latency or distance moved in ArKO mice, contrasts with some other studies where estrogen has been shown to facilitate watermaze learning. For example, estradiol treatment alleviated memory deficits in aged female mice [9] and ovariectomy caused impairment of spatial reference memory [7]. On the other hand, others have shown that acute estrogen treatment actually worsened watermaze performance [3], while ovariectomy did not affect watermaze learning [33]. Interestingly, this latter studies also suggested that NMDA receptor function and estrogen effects on learning and memory were unrelated. One reason for the apparent contradiction between our data and some literature reports is, that we used ArKO mice. In this model, estrogen deficiency may have other consequences on behaviour than the widely used ovariectomy approach. In contrast to ovariectomy, in ArKO mice, testosterone levels are not reduced and brain estrogen production is absent [8,23].

In contrast to watermaze learning, we have previously reported that both male and female ArKO mice performed significantly worse than WT controls in a Y-maze test for short-term spatial reference memory [16]. The Y-maze test does not depend on learning a new behaviour or rule but relies on an innate tendency of a mouse to explore a novel environment [5]. On the other hand, the Morris Watermaze test requires the animal to learn and remember the location of the hidden platform. This test is especially sensitive to hippocampal damage, and reflects attention, memory, and learning strategy [19]. Therefore, the inference we can draw from these data is that estrogen differentially influences different cognitive functions.

It is furthermore important to note, that estrogen deficiency (and the associated increase in testosterone levels in the ArKO mice [8]) may interact with several other neurotransmitter systems that are involved in learning and memory. For example, ArKO mice show increased serotonergic activity in the hippocampus [4], which is intricately involved in memory [15,19]. Similarly, in ArKO mice, there is evidence for increased dopaminergic activity involved in locomotor activity [30] and for apoptosis in dopaminergic neurons of the hypothalamus [10]. Further studies are required to assess the importance of the neurotransmitter changes in estrogen-deficient ArKO mice for learning and memory.

In conclusion, this study shows that in the absence of estrogen, the female ArKO mice exhibit better performance

during the free-trials in the watermaze test. This could be related to the higher levels of NMDA receptor subunit transcripts levels in the ArKO hippocampus as compared to the WT.

Acknowledgments

This work was supported by USPHS grant R37-AG 08174. Dr. M. van den Buuse was supported by the Griffith Senior Research Fellowship of the University of Melbourne, Australia. The Mental Health Research Institute of Victoria is a Stanley Research Centre, supported by the Stanley Medical Research Institute, Bethesda, MD, USA.

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