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Original Article

Impairment of prenatal exposure to MRI on rats' spatial memory is associated with CaMK IIβ

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Abstract: To investigate the effects of MR prenatal exposure on the ability of learning and memory, we examined the behavior changes of rats after treatment and its molecular mechanism. Fourteen pregnant Sprague-Dawley rats were randomly divided into an experimental group and a control group, each with 7 mother rats. The experimental animals were exposed to MR between days 12 and 18 of gestation for 10 min a day. After birth, all male litters were randomly divided into an MRI-scanning group (experimental group) and a control group. And Morris Water Maze (MWM) tasks were tested at 1st-, 2nd- and 5th-month after birth. The results showed that the MR exposed rats at the age of 2 months spent less time than the non-exposed ones in the platform quadrant during probe trial test, means a minor "reference memory" deficit for the treatment animals. Then the 2 months groups were selected for microarray and Western blot to detect the molecular mechanism of behavior deficit. Here we found that only 12 genes among all the tested were up or down-regulated. Western bolt showed that CaMK IIβ was changed significantly. We suggested that the potential impairment of spatial learning and memory induced by prenatal MR exposure was aged dependence, which may mediated by regulation of CaMK IIβ.

Keywords: Prenatal exposure, magnetic resonance imaging (MRI), morris water maze (MWM), microarray, reference memory

Introduction

It is well known that the development of an embryo is a high sensitive process and the consecutive stages involved can be easily disturbed. Several line evidence demonstrated that exposure of pregnant women to certain conditions might affect their offspring's development of learning, behavioral and/or mood disorders [1, 2]. Magnetic resonance imaging (MRI) has become important diagnostic tools during pregnancy because of their noninvasive and no ionizing character [3, 4]. The safety and risk assessments are concerned by pregnancy woman who accepted MRI examination. However, no consensus result has emerged on the potential hazards of MRI though previous papers which addressed some of the key safety issues about it. Heinrichs *et al*. found a slight but significant reduction in fetal crown-rump length after exposure of mice to MRI which conditions are equivalent to those used in human MRI at field strength of 0.35 T [5]. In another study using the same MRI exposure conditions, Tyndall observed an increased fetal resorption rate and a significant increase in eye malformation [6]. Later, it is reported that there aren't harmful effects of magnetic exposure at 1.5 T in utero during pregnancy on neurological, social, and motor development at 3 months of age [7]. In rats, graded effects on behavior were observed after exposure to 7 and 14 T magnetic fields MRI [8] and the development of new systems at higher magnetic fields raises issues of potential health hazards [9]. However, there are reported that repetitive exposure of mice to strong static magnetic fields in utero does not impair the basal emotional and cognitive behavior, as well as fertility in adulthood [10, 11].

It has been shown that when subjected to an insult (ischemia, hypoglycemia and epilepsy), brain damage is not homogenous [12], with the hippocampal formation being one of the most vulnerable regions [13]. And extensive evidence has indicated that the hippocampal formation is a critical brain area for spatial learning and memory [14]. Our previous study using 0.35 T MRI didn't detect the impairment spatial learn-

ing and memory in male offsprings prenatal exposure magnetic fields at their 2-month age [15]. Here, using stronger magnetic fields, However, to our knowledge, the stronger the influence of magnetic fields on spatial learning and memory in offsprings who exposure to prenatal MR haven't been investigated.

Morris water maze (MWM) is a well-established setup to test the spatial learning and memory ability of rodents [16], in which, the animals learn to locate a submerged platform using spatial environmental cues. This ability is believed to require at least the hippocampal formation [17]. Therefore, MWM performance has been broadly used to detect the impairment of learning and memory owing to developmental disorders in rodents.

Evidences accumulated indicating that during the process of learning and memory, complex signaling cascades of biochemical changes are activated in the neurons and neurogliocytes [18]. Many of these changes alter the expression of genes, whose protein products contribute to behavioral physiology. Molecular genetics provides the techniques to identify the genes involved in a particular behavior and to determine how the proteins they encode control the related behavior [19]. So far, even though the effects of MRI exposure on the behavior, fetal development, and/or malformations were emphasized, but there are few of studies to probe molecular mechanism [18, 20]. The Gene Chip Rat Neurobiology U34 Array included over 1,200 sequences relevant to neurobiologic processes such as learning and memory. Thus it is a powerful tool for generating a focused set of data specific for the study of neurobiology. In the present study, we detected firstly if the offspring show deficit in spatial; learning and memory behavior after exposing to MR during pregnancy; then, using microarray technique, we examined changes of genes expression in hippocampus of offspring's brain after prenatal MR exposure; last, the changes of immunoreactive proteins related to hippocampal functions encoded by genes identified by microarray test were detected.

Materials and methods

Animals

Fourteen healthy pregnant Sprague-Dawley rats were used, among them 7 pregnancy rats for experimental group and the other 7 ones for control group. Each of the 14 pregnant rats was housed in a standard plastic cage (46 × 31.5 × 20 cm) with food and water available *ad libitum*. The room temperature was maintained at 21 ± 1°C. The light-dark cycle was 12 h (hour): 12 h (hour) (light on at 7:00 a.m.). All of the animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the procedures were approved by the Local Animal Care and Use Committee.

MRI field exposure

The experimental pregnant rats were exposed to MRI between days 12 and 18 of gestation for 10 min a day starting at 7 p.m. The control group pregnant rats were exposed to a sham control field under similar conditions. The MRI field we used was composed of three magnetic fields: 1.5 T static magnetic field, 1.0 kW power-pulsed magnetic field, and grads magnetic field produced by a Gyroscan Intera (Philips, Holland). The field was identical to that used in human clinical scanning situations. The MRI magnetic field used a spin echo technique. T1 had a time rate (TR) of 525 ms and a time echo (TE) of 13 ms, with a slice thickness of 7 mm, number of signal averages (NSA) 5; T2 had a TR of 1800 ms and a TE of 350 ms, with a slice thickness of 7 mm, NSA 3. The condition (1.5 T MRI and 10 min) were selected according to previous study [9], routing clinical using, in which the patient exposure is at a 1-2 T for a shorter time interval (10-40 min).

Morris water maze task

At postnatal day 21, all the male pups, from both the experimental and control groups, were separated from their mothers and assigned to two general groups: MRI-scanning group (experimental group), and control group. In each group, rats were randomly divided into three testing-age subgroups (designated according to their age at MWM testing: 1, 2, and 5-month subgroups). In total, 47 offspring rats were assigned into two general groups and 6 subgroups, and a maximum of two rats per litter were assigned to each subgroup. All the subgroups (named groups later) contained 8 rats each, besides the control 5-month group composed of 7 rats.

The water maze was a circular pool tank (diameter: 120 cm; height: 55 cm) filled with water to a depth of 41 cm, with a temperature of 23 ± 1°C. A round transparent platform (8 cm in diameter) was placed at the center of a designated quadrant and submerged 1 cm beneath the water's surface. The distance from the platform center to the pool edge was 35 cm. Rats were tested for 5 successive days. Two test sessions were performed on each of the first 4 days, and one session was conducted on the last day. Each session consisted of four trials, with an inter-trial interval of 60 s (second). The inter-session interval within each test day was 2 h. Maze training was conducted between 8:00 and 12:00 a.m., and between 2:00 and 6:00 p.m. In each trial, the rat was gently placed into the pool at the middle site of the circular edge at each quadrant, arbitrarily defined as east, south, west, and north. The animal was allowed 120 s to swim to the hidden platform, and then left on the platform for at least 3 s. If the rat could not locate the platform within 120 s, it would be gently guided to the platform by the experimenter and then left on it for 10 s, and its performance score (latency) was marked as "120 s". MWM behavior was videotaped via a commercial video/computer system (Beijing Logon Science and Technology). The final test (probe trial) was performed on the afternoon of the 5th training day, during which the platform was removed from the maze. During the probe trial the animal was released into the quadrant opposite to the one that had previously contained the platform (the "platform" quadrant), and allowed to swim in the maze for 2 min. The video/computer system automatically recorded the latency for finding the platform, the time spent in the platform quadrant, and the number of crossings over the target area for each rat.

Animal dissection and RNA extraction

Because only the 2-month aged MRI treatment rats showed a minor behavior deficit (see results), then both the experimental and control rats at this age were selected to be used in the microarray test. On the next day after the last test session, all the 16 rats of the two groups were decapitated and the hippocampal formations were removed on an ice-cooled stage and then frozen in liquid nitrogen.

Total RNA was extracted from individual rat hippocampal formation in experimental and control groups with Trizol reagent (Invitrogen, Carlsbad, CA), and the mRNA was purified with an Oligotex mRNA kit (Qiagen, Hilden, Germany). Our lowest A_{260}/A_{280} ratio used in this procedure was 2.0, and agarose gel separation of total RNA by this procedure confirmed extraction of a high yield of intact total RNA. To minimize sample-to-sample variations and increase the quantity, the RNA for each experiment was prepared from equal amounts of total RNA pooled from hippocampus of experimental and control group, each has 8 animals. Thus, RNA sample pools (MRI RNA samples and control RNA samples) were generated by mixing equal amounts of total RNA extracted from individual rat's hippocampal formation.

Array hybridization and scanning

Twenty micrograms of total RNA were used from each sample, and probe preparation and hybridization with U34 Rat Neurobiology Gene Chip oligonucleotide array, which contains 1322 functional genes, with the protocol outlined in the Gene Chip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). The Gene Chip array was then washed twice and stained with a streptavidin-phycoerythrin conjugate in the Affymetrix Fluidics Station. To reduce hybridization errors, a competitive hybridization method was employed, and then hybridizations for each RNA sample were duplicated. The oligonucleotide gene chip used in the present study contains several probe sets specific for rat housekeeping genes (β-actin, GAPDH), which served as internal controls, also GAPDH were selected as the internal controls for RT-PCR and Western blot, as exercise did not alter their expression [21].

Real-time PCR

To verify the changes in expression determined by the microarray analysis, three genes were choosing to do quantitative RT-PCR based on SYBR Green (Applied Bio systems) that was used to verify the results of microarray analysis, primer sequences used for real time RT-PCR were listed in **Table 1**. For each specific gene tested, cDNAs from 5 μg of total RNA from each sample were generated for real-time PCR amplification. 20 μl of PCR reaction mixture contained 12.5 μl of 2 × concentration SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Each sample was subjected to 45 cycles of real time PCR (ABI 7000; Applied Biosystems) where fluorescence was measured several times during each cycle of two-step PCR alternating between 95°C for 15 secs and 59°C for 1 min. To calculate a relative

Table 1. Primer sequence for real time RT-PCR

Gene	Forward primer	Reverse primer
Af000423	CCCTTTTGCTGTCACCTCTCA	CCCACACATCCCATTCTCAAC
M16112	CCATAGAGGACGAAGATGCCA	CAGATTTTCGCATAGGCCTCA
U69109	ACCTGGCAGAGCTCATCAACA	TGGCTTGGTCTACAGCATCCA

amount of transcript present for each gene, the expression value of each gene was normalized to the amount of GAPDH in that sample. The normalized expression values for all control and treated samples were averaged, and an average fold change was determined. Each condition was acquired in triplicate at least. A Student's t test was conducted between the normalized relative expression values for each control and treated samples to determine statistical relevance.

Western blot

Hippocampal formation from the second set of identically treated animals was stored with liquid nitrogen and used to detect if there were protein changes of the MAP-1A and CaMK II, which gene changes were found in the microarray test (see results). The hippocampal formations were homogenized with ice-cold lysis buffer consisting of 20 mM Tris, 30 mM NaCL, 10 mM Na_3VO_4, 1 mM Na_2EDTA, 50 mM NaF, 1% NP-40, 0.5% deoxycholace sodium, 10 mM leupeptin, 10 mM aprotinin, 10 mM $Na_4P_2O_7$, 1% SDS, 1 mM PMSF, and pH7.5. The homogenates were centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was used for analysis. Protein concentration was determined by the method of Bradford (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as standard sample. Protein samples (40 μg) were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris -buffered saline containing 0.1% Tween 20 (TBST), and then probed with antibodies against MAP-1A (Santa Cruz Biotechnology) and CaMK II (Cell Signaling Technology). After incubation with secondary antibodies, immunoblots were visualized with the Quantitive one software (Bio-Rad, Hercules, CA, USA).

Cell immunohistochemical staining (IHC)

The treated tissues were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton X-100. Then the fixed cells were incubated with MAP-1A, and CaMK II primary antibody followed by streptavidin peroxidase-conjugated secondary antibody (SABC method). Using previously reported immunohistochemical techniques, the staining was visualized by using diaminobenzidine and counterstained with hematoxylin, ten independent high-magnification fields (× 400) were evaluated for each section using a laser scanning confocal microscope (TCS2 SP5; Leica Microsystems, Wetzlar, Germany).

Data analysis and statistics

Data are presented as the mean ± SD and performed at least three independent replicates. SPSS software, 16.0 (SPSS, Inc, Chicago, IL, USA) and Graphpad Prism 6.0 (CA, USA) were used for a two-tailed Student t-test to evaluate the statistical significance. Differences were defined as $P < 0.05$.

Results

The prenatal-magnetic-fields-exposed rats in early adulthood spent less time in the platform quadrant than the non-exposed rats

As shown in **Figure 1**, two way repeated ANOVN analysis showed that the latencies of finding the platform for exposed group of 2-month age showed a trend increase compare to their control group during the acquisition test ($F [1,14] = 3.463$, $P = 0.084$). However, there is no significant difference for other aged groups ($F [1,14] = 2.111$, $P = 0.168$ for 1 month and $F [1,13] = 0.758$, $P = 0.400$ for 5 months respectively).

As shown in **Figure 2**, two way repeated ANOVN analysis showed that there was significant difference in the time spent in the platform quadrant for different groups during the probe trial between exposed and unexposed group of 2-month age ($F [1,14] = 6.388$, $P = 0.025$). No significant effects of the exposure were found for the remaining groups.

Differential gene profile in the hippocampus of rats after prenatal exposure to magnetic fields

Of the 1300 genes on the microarray, only 12 genes were changed in the MRI exposure group compared to untreated controls. Among the 12

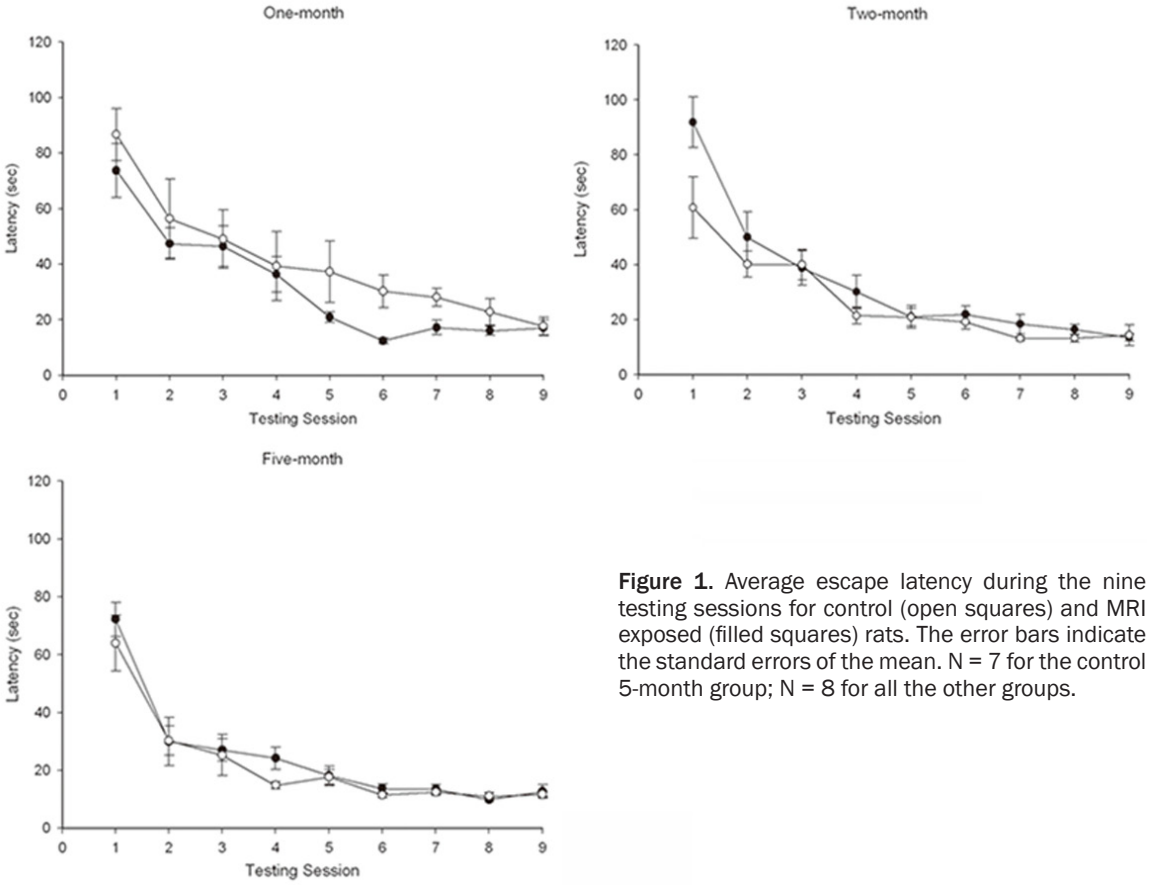

Figure 1. Average escape latency during the nine testing sessions for control (open squares) and MRI exposed (filled squares) rats. The error bars indicate the standard errors of the mean. N = 7 for the control 5-month group; N = 8 for all the other groups.

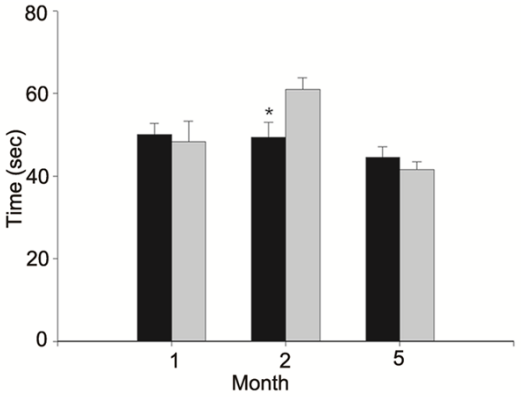

Figure 2. Average time spent in the platform quadrant for control (gray bars) and MRI exposed (black bars) rats in the probe tests. The error bars indicate the standard errors of the mean. *Indicating the significant differences between control and MRI exposed groups (*$P < 0.05$ by t test). $N = 7$ for the control 5-month group; $N = 8$ for all the other groups.

genes, ten were down regulated (indicated as negative values; fold = -1.3 to -2.1), while other two were unregulated (fold = 1.3). The genes changed after MRI exposure and their functions were listed in **Table 2**. Results obtained with high density oligonucleotide microarrays indicated that exposure of rat fetuses developing in utero to MRI obviously influenced approximately 1% of genes' expression among all the genes on the chip. This ratio is very low comparing to other papers that also used with Affymetrix Rat Neurobiology U34 arrays [22]. Among all the down regulated genes, we were more interested in CaMK IIβ, synaptotagmin 11 and protein tyrosine kinase 2 beta, and selected them to do RT-PCR, because they were involved in synaptic transmission or learning and memory.

Confirmation of the expression differences by real-time RT-PCR

Using Real-Time PCR, three genes described above were selected to validate our microarray data: Af000423, M16112, and U69109. The housekeeping gene GAPDH was used to normalize both microarray and Real-Time PCR

Table 2. Changes in gene expression after MRI exposure

Accession number	Gene name	Fold change	Function
Down regulated			
AF000423_at	Synaptotagmin 11	-1.7	Integral to plasma membrane; transporter activity
AF058795_at	G protein-coupled receptor 51	-1.5	G-protein coupled receptor activity; GABA-B receptor activity; metabotropic glutamate
L27421_at	Frequenin homolog (Drosophila)	-2.1	Calcium ion binding
M16112_at	Calcium/calmodulin-dependent protein kinase II beta subunit	-1.4	Protein kinase activity; protein serine/threonine kinase activity; calmodulin binding
M28648_s_at	ATPase, Na^+/K^+ transporting, alpha 3 polypeptide	-1.4	Sodium/potassium-exchanging ATPase activity
M83196_at	Microtubule-associated protein 1A	-1.5	Microtubule stabilization
rc_AI045501_s_at	Neuronal pentraxin receptor	-1.5	Integral to membrane
S61973_at	NMDA receptor glutamate-binding chain	-1.4	N-methyl-D-aspartate selective glutamate receptor activity
U08290_at	Neuronatin	-1.3	Development
U69109_s_at	Protein tyrosine kinase 2 beta	-1.3	Protein kinase activity
Up regulated			
D00688_s_at	Monoamine oxidase A	1.3	Amine oxidase activity
E13644cds_s_at	Protein carrying the RING-H2 sequence motif	1.3	Neurogenesis

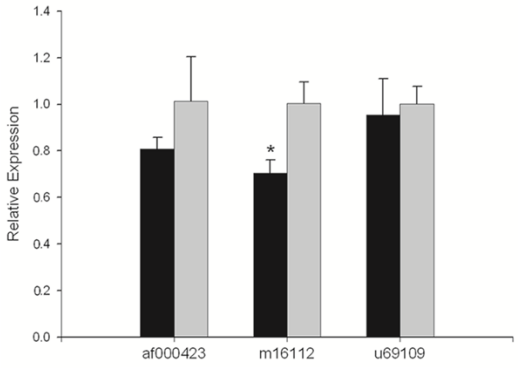

Figure 3. Comparison of the gene expression difference between control (gray bars) and MRI exposed (black bars) 2-month rats using real-time PCR. Results are shown using expression relative to the control for each gene after normalization to GAPDH. *Indicating the significant differences between control and MRI exposed groups (*$P < 0.05$ by *t* test).

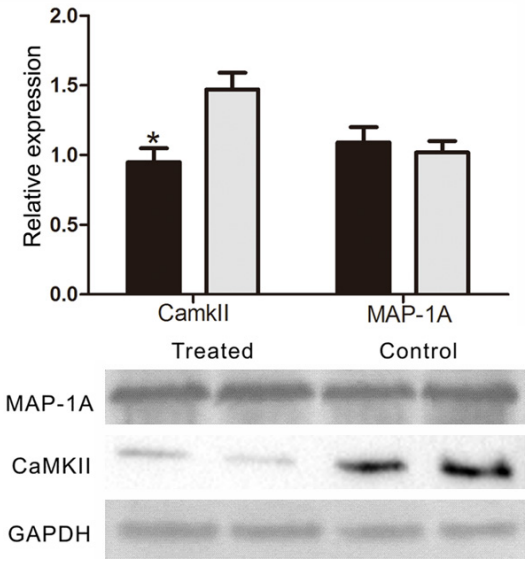

Figure 4. Comparison of the protein expression difference between control (gray bars) and MRI exposed (black bars) 2-month rats using western blot (*$P < 0.05$ by *t* test).

data. The results presented in **Figure 3** show that the expression patterns obtained with Real-Time PCR was consistent with the microarray results. However, only CaMK IIβ, but the other two genes, was different significantly between control and exposure group samples.

Protein changes for CaMK II and MAP-1A

In order to study the molecular mechanisms of age-specific effect of prenatal exposure to MRI on the spatial memory, it is important to determine the changes of functional proteins in addition to the genes expression. Therefore, we tested the target proteins, CaMK II and MAP-1A, identified by hybridization analysis. Specific immunoblot were performed on equal amounts of total protein from individual samples of each animal. The results showed similar results in **Figure 4**. For MAP-1A, though there was a difference in microarray, no changes were found in RT-PCR and western blot. And for CaMK II, the protein level were also down-regulated in the treated group. In addition, we performed immunohistochemical staining to confirm the data, the results revealed the same phenomenon that the expression of MAP-1A had no significant change, however, the CaMK II expression decreased obviously after the treatment (**Figure 5**).

Discussion

In the present study, we employed the reference memory version of the MWM, in which the animal's ability to use environmental cues to locate the former position of a submerged escape platform in space during the trial is measured [23]. Reference memory acquisition in the MWM involves hidden-platform acquisition training and probe trail testing. The first process involves learning the procedure, which is learning to search for the platform using the cues in the environment. And then the spatial accuracy of the animal is determined during a probe trial [16]. Our previous study using 0.35 T MRI didn't detect the impairment spatial learning and memory in male offsprings prenatal exposure magnetic fields at their 2 month age [15]. Here, using stronger magnetic fields, we found a slight and recoverable impairment at same age stage. Interestingly, we found that spatial learning and memory wasn't impaired at early development (1-month age), which is consistent with previous human study using the same strong magnetic field. They reported that no harmful effects of prenatal MR exposure in pregnancy are detected at 3 months age [7]. Though adverse influences on embryonic development induced by weren't much severe, it should be noted the potential effects on spatial learning and memory especially under the strong MRI.

Behavior is the result of the interaction between genes and the environment. The genes expressed in neurons encode proteins that are important for development, maintenance and

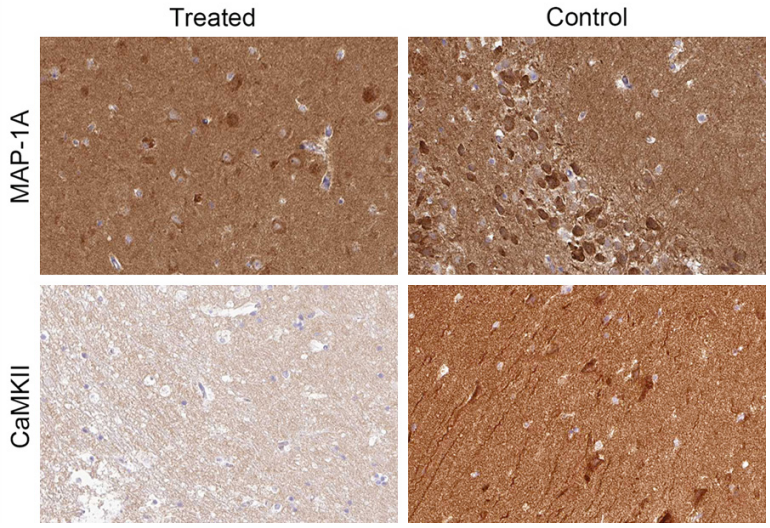

Figure 5. The representative picture of immunochemical staining of serial hippocampal sections for the protein expression difference between control and MRI exposed 2-month rats.

regulation of the neural circuits that underline all aspect of Learning and memory [19]. A complex network of brain systems and serial and parallel molecular events involve in the process of learning and memory. Different chains of molecular cascades contribute to the events, for example, glutamate receptors; CaMK II, PKA, PKC and ERK1/2 are all necessary for LTM formation [22, 24-26]. Using Gene Chip Rat Neurobiology U34 Array, we detected 12 genes differential expression gene after prenatal magnetic fields exposure and three of them related to function of hippocampus were confirmed by RT-PCR. Among of them, we were considered interesting in *CaMK II* and *MAP-1A* due to they are the most closely related to learning and memory. CaMK II play an important role in learning and memory, as well as in formation of LTP. MAP-1A has been proposed to be involved in regulating the interaction between axonal microtubules and actin filaments, which is believed to be essential for neuronal morphogenesis and function.

Proteins that are newly synthesized during memory consolidation may contribute to restructuring processes at the synapse and thereby alter the efficiency of synaptic transmission. We further investigate the protein changed encoded by *CaMK II*, *CaMK IIβ* and *MAP-1A*. No changes were found except for CaMK IIβ. It is known that some apparent changes in mRNA were not reflected at the level of protein, this could be the result that some changes in mRNA may be subject to additional regulation at the levels of translation and degradation. In present study since the genes were not changed highly, there were no obvious variations appeared in their protein or/and mRNA levels during translation or/and post-translational modification.

CaMK IIβ belong to a family of high conserved serine/threonine kinase, which in mammals encoded by four different genes, *Camk2a, Camk2b, Camk2d* and *Camk2g*, giving rise to four isozymes, CaMK IIα, CaMK IIβ, CaMK IIδ and CaMK IIγ kinase type [27]. Recent studies found that CaMK IIβ regulated oligodendrocyte maturation and CNS myelination [28]. Further study reported that CaMK IIβ drive dendrite retraction and pruning [29]. Our result indicated that associations between CaMK IIβ and spatial learning and memory for the first time. Combine with previous studies, we suggested that prenatal MR exposure regulated the expression of CaMK IIβ, which may influence the development of dendrite and then impairment spatial learning and memory. In summary, we revealed the potential impairment of spatial learning and memory in adulthood at aged dependence, which may mediated by regulation of CaMK IIβ.

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Disclosure of conflict of interest

None.

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