

## Effects of maternal enflurane exposure on NR2B expression in the hippocampus of their offspring

Fo-Quan Luo<sup>1,\*</sup>, Jun-Wu Liu<sup>2</sup>, Shu-Xin Tang<sup>3</sup>, Wei-Lu Zhao<sup>1</sup>, Yan Hu<sup>1</sup>, Lin Xu<sup>1</sup>, Meng-Yuan Li<sup>1</sup>

<sup>1</sup>Department of Anaesthesiology, the First Affiliated Hospital of Nanchang University, Nanchang, China, <sup>2</sup>Department of Anaesthesiology, the People's Hospital of Meishan, Meishan, China, <sup>3</sup>Department of Medical Library of Nanchang University, Nanchang, China

This work aims to study the pathogenesis of learning and memory impairment in offspring rats resulting from maternal enflurane anesthesia by focusing on the expression of the *N*-methyl-D-aspartic acid receptor subunit 2B (NR2B) in the hippocampus of the offspring. Thirty female Sprague-Dawley rats were randomly divided into three groups: control (C group), 4 h enflurane exposure (E1 group), and 8 h enflurane exposure (E2 group) groups. Eight to ten days after the initiation of pregnancy, rats from the E1 and E2 groups were allowed to inhale 1.7% enflurane in 2 L/min oxygen for 4 h and 8 h, respectively. Rats from the C group were allowed to inhale 2 L/min of oxygen only. The Morris water maze was used to assay the learning and memory function of the offspring on postnatal days 20 and 30. RT-PCR and immunohistochemistry assays were then used to measure the mRNA levels and protein expression of NR2B, respectively. Relative to offspring rats from the C group, those from the E1 and E2 groups exhibited longer escape latencies, lesser number of crossings over the platform, and less time spent in the target quadrant in the spatial exploration test ( $P < 0.05$ ). In addition, the mRNA and protein expression levels of NR2B in the hippocampus of offspring rats in the E1 and E2 groups were down-regulated ( $P < 0.05$ ). No significant differences between the E1 and E2 groups were observed ( $P > 0.05$ ) in terms of mRNA levels and protein expression of NR2B. The cognitive function of the offspring is impaired when maternal rats are exposed to enflurane during early pregnancy. A possible mechanism of this effect is related to the down-regulation of NR2B expression.

**Uniterms:** Enflurane/use/maternal anesthesia. Enflurane/adverse effects/experimental study. Enflurane/effects/pregnancy. *N*-methyl-D-aspartic acid/receptor/gene expression.

Este trabalho objetiva o estudo da patogênese de deficiência no aprendizado e memória de prole de ratos resultante da anestesia maternal por enflurano, por meio da expressão da subunidade 2B do receptor do ácido *N*-metil-D-aspartico (NR2B) no hipocampo dos filhotes. Dividiram-se, aleatoriamente, 30 fêmeas de ratos Sprague-Dawley em três grupos: controle (grupo C), exposição ao enflurano por 4 h (grupo E1) e por 8 h (grupo E2). De oito a 10 dias após o início da gravidez, os ratos dos grupos E1 e E2 inalaram enflurano 1,7% em 2 L/min de oxigênio, por 4 h e 8 h, respectivamente. Ratos do grupo C inalaram apenas 2 L/min de oxigênio. O labirinto de água de Morris foi empregado para analisar as funções de aprendizado e memória da cria em 20 e 30 dias após o nascimento. Utilizaram-se ensaios de RT-PCR e de imuno-histoquímica para medir os níveis de mRNA e expressão da proteína do NR2B, respectivamente. Em comparação com os ratos controle do grupo C, aqueles dos grupos E1 e E2 exibiram latências de escape mais longas, menor número de travessias na plataforma e menos tempo gasto no quadrante alvo no teste de exploração espacial ( $P < 0,05$ ). Adicionalmente, os níveis de expressão de mRNA e de proteína do NR2B no hipocampo dos filhotes nos grupos E1 e E2 estavam reduzidos ( $P < 0,05$ ). Não se observaram diferenças significativas entre os grupos E1 e E2 ( $P < 0,05$ ) quanto aos níveis de mRNA e à expressão de proteína de NR2B. A função cognitiva dos filhotes é prejudicada quando as mães são expostas ao enflurano durante o início da gravidez. O mecanismo possível para esse efeito está relacionado à diminuição na expressão de NR2B.

**Unitermos:** Enflurano/uso/anestesia maternal. Enflurano/efeitos adversos/estudo experimental. Enflurano/efeito/gravidez. Receptor do ácido *N*-metil-D-aspartico/expressão gênica. Ácido *N*-metil-D-aspartico/receptor/expressão gênica.

\*Correspondence: Fo-Quan Luo. Department of Anaesthesiology. The First Affiliated Hospital of Nanchang University. Nanchang 330000 - China. E-mail: foquanluo@163.com

## INTRODUCTION

Certain general anesthetics have been found to induce neuronal apoptosis in the brain, leading to permanent impairments of spatial learning. Clinical studies have confirmed that some patients suffer from long-term cognitive impairment after administration of general anesthesia. For example, the common clinical anesthetic enflurane induces neuronal apoptosis (Brown, Purdon, Van Dort, 2011; Reddy, 2012) and may cause postoperative cognitive impairment in children after inhalation (Lei, Guo, Zhang, 2012).

Early pregnancy is an important phase in the development of the mammalian brain and as such, during this phase the brain is vulnerable to various factors. The incidence of non-obstetric surgery during pregnancy has been estimated to range from 1.0% to 2% (Grewal, 2010). Enflurane may enter the embryo through the placental barrier. However, the effects of maternal enflurane anesthesia during early pregnancy on the learning and memory of the offspring are yet to be reported. Therefore, examining this issue is of great significance and could provide an experimental basis for the clinical selection of anesthesia. In a previous study, we demonstrated that exposure to enflurane anesthesia during early pregnancy can reduce the cognitive capacity of rat offsprings; however, the exact mechanism of this action remains unclear.

The *N*-methyl-D-aspartate receptor (NMDAR) subunit 2B (NR2B) is highly expressed in the hippocampus and closely related to changes in cognitive function. Increases in NR2B expression have been found to produce stronger long-term potentiation (LTP) in presynaptic neurons in the CA1 region of the hippocampus of rats, thereby enhancing spatial learning and memory, as well as fear memory (Zhuo, 2009). The expression level of NR2B in the hippocampal CA1 region decreases with age. Furthermore, NR2B expression in juvenile, adult, and aged rats corresponds to differences in spatial memory function. As such, decreases in NR2B expression in rats are also closely related to impairment of spatial learning. Rammes *et al.* (2009) found that the NR2B-selective antagonist ifenprodil or RO25-6981 can significantly inhibit improvements in cognitive function and enhancements of LTP induced by isoflurane. Isoflurane specifically induces up-regulation of NR2B expression in the hippocampus and enhances LTP in neurons in the CA1 area, thereby improving learning ability. These results indicate that NR2B has a close relationship with learning and memory.

The present study evaluates the effects of maternal enflurane anesthesia exposure on NR2B expression in

the hippocampus of Sprague-Dawley (SD) rat offspring in order to explore the pathogenesis of a demonstrated impairment in learning and memory.

## MATERIAL AND METHODS

### Animals

Thirty male and thirty female SD rats (14 weeks-old, 200 - 300 g in weight) were provided by the Animal Science department of the medical college of Nanchang University. The Morris water maze (ZH0065, Beijing Sunny Instruments Co., Ltd.) was used to assess spatial learning and memory. Based on the results of the task, male and female rats were divided into three groups of breeding pairs, each group including 10 male and 10 female rats. No significant difference was observed among the groups in their performance in the Morris water maze. Eight to ten days after conception, pregnant rats in the E1 and E2 groups were placed in an anesthetic box (size: 40 × 40 × 25 cm) and treated with 1.7% enflurane (Abbott Pharmaceutical Co., Ltd., Shanghai, 78195TL) in 2 L/min oxygen for 4 and 8 h, respectively. Rats in the C group were treated with 2 L/min oxygen only. A noninvasive ECG monitor was used to monitor the heart rate, breath rate, blood oxygen saturation, and blood pressure of the rats. Any rats that experienced hypoxia (under 95% blood oxygen saturation) or hypotension (decrease in systolic blood pressure to less than 20% of baseline) were excluded from our study, and another dam was selected to replace them and maintain the sample size. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Hospital of China Medical University.

### Morris water maze test

A Morris water maze system was used to assess the cognitive function of the offspring rats daily for 6 days (one trial per day) starting at postnatal day 20 and again at postnatal day 30. The behavioral experiments began at 9:00 AM every day. Prior to the formal experiment, all rats were trained to swim to the hidden platform and given 120 s to search for the platform in a test room. Rats that failed to find the platform within the given time were guided to the platform and allowed to stay on it for 60 s. In the formal trial, rats were placed in the water in a different quadrant and the time taken to find the platform was recorded as the search time (also called the escape latency). If a rat failed

to find the platform within 120 s, this was recorded as the search time. On the seventh day, the hidden platform was removed from the tank, and a spatial exploration trial was performed. We recorded the frequency at which the rats crossed the previous location of the platform within 120 s and the time that the rats swam within the target quadrant that had contained the platform. During testing, we ensured consistent environmental variables, such as lighting, to eliminate environmental interference.

### Sample preparation

After water maze testing was complete, all offspring rats were sacrificed. The right hippocampus was dissected under sterile conditions and placed into an Eppendorf tube treated with water containing 0.1% diethylpyrocarbonate and immediately stored in a liquid nitrogen container. The left hippocampus was fixed with 4% paraformaldehyde for 24 h to produce paraffin sections.

### RT-PCR assay

A Total RNA kit (Promega Co., Ltd., USA) was used to extract total RNA from the right hippocampus. cDNA was synthesized by means of reverse transcription using an RT kit (Promega Co., Ltd.). The primer sequences were as follows: NR2B forward primer 5'-GACCACTATCACCATCAC-3', reverse primer 5'-AGAGAACTTGCCATACAG-3', 256 bp;  $\beta$ -actin forward primer 5'-TCAGGTCATCACTATCGGCAAT-3', reverse primer 5'-AAAGAAAGGGTGTAACGCA-3', 432 bp (Shanghai SANGON Biological Engineering Co., Ltd.). The PCR reaction system was as follows: 12.5  $\mu$ L 2 $\times$  PCR TaqMix, 1  $\mu$ L forward primer, 1  $\mu$ L reverse primer, and 2  $\mu$ L cDNA template were added to 25  $\mu$ L of water. PCR conditions were as follows: 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 55.5 °C for 1 min, and 72 °C for 1 min. It was then held at 72 °C for 5 min after cycling. The amplified products were visualized with UVP Bio Spectrum using 1.5% agarose gel electrophoresis. We used the Quantity One software to detect the optical density (OD) of all strips and analyzed the mRNA expression of NR2B using the relative OD value ( $OD_{NR2B}/OD_{\beta-actin}$ ).

### Immunohistochemistry assay

The hippocampus tissue was embedded in paraffin. Each tissue sample was cut into sections of equal thickness (about 4  $\mu$ m). One section from every ten was selected for immunohistochemical analysis.

Immunohistochemical staining was performed using an immunohistochemistry kit according to the manufacturer's instructions. The primary antibody was rabbit anti-rat NR2B (1:200, Abcam Company, USA), and the secondary antibody was horseradish peroxidase labeled anti-rabbit IgG (Beijing ZSGB-Bio Company). An HMIAS-200W analysis and management system (Wuhan Champion Image Ltd.) with the capability to produce high-resolution color pictures was used to collect pictures and analyze the OD value.

### Statistical analysis

Statistical analysis was performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA), and all results are presented as mean  $\pm$  SD. One-way analysis of variance was used to compare different groups. A *P* value <0.05 denoted a significant statistical difference.

## RESULTS

### Morris water maze test

The Morris water maze test task showed that the escape latency of the three groups decreased with the increase of training times, indicating that the rats were capable of learning the platform location. Compared with the C group, rats from the E1 and E2 groups exhibited a longer escape latency ( $P < 0.05$ ) (Table I), a lower number of crossings over the platform ( $P < 0.05$ ) (Table II), and less time spent in the target quadrant during the spatial exploration test ( $P < 0.05$ ). These results indicate that the spatial learning and memory of offspring rats in the E1 and E2 groups was impaired relative to rats in the C group. This suggests that exposing pregnant SD rats to enflurane could impair spatial learning and memory in their offspring. However, no significant differences between the E1 and E2 groups were observed. This result implies that spatial learning and memory is impaired by maternal enflurane exposure in a manner independent of exposure time.

### RT-PCR assay

At postnatal day 20, NR2B mRNA levels in the hippocampus of rats in the E1 and E2 groups were  $0.64 \pm 0.07$  and  $0.63 \pm 0.07$ , respectively; significantly lower than that of rats in the C group ( $0.71 \pm 0.07$ ) ( $P < 0.05$ ). At postnatal day 30, NR2B mRNA levels in the hippocampus of rats in the E1 and E2 groups were also significantly lower than that of rats in the C group ( $P < 0.05$ ). Together with the Morris water maze test results, these findings

**TABLE I** - Comparisons of escape latency among Control group, E1 group and E2 group

Time	Group	Testing day					
		1st day	2nd day	3rd day	4th day	5th day	6th day
postnatal day 20	C group	109±16	103±16	94±26	74±19	76±26	64±15
	E <sub>1</sub> group	112±12	107±23	115±7 <sup>a</sup>	100±15 <sup>a</sup>	96±11 <sup>a</sup>	77±15
	E <sub>2</sub> group	113±9	106±20	116±9 <sup>a</sup>	103±12 <sup>a</sup>	100±12 <sup>a</sup>	80±18
postnatal day 30	C group	115±8	114±8	101±14	85±19	78±18	76±26
	E <sub>1</sub> group	111±13	114±9	111±10 <sup>a</sup>	105±7 <sup>a</sup>	96±10 <sup>a</sup>	80±14
	E <sub>2</sub> group	114±9	113±11	112±12 <sup>a</sup>	111±8 <sup>a</sup>	98±13 <sup>a</sup>	83±20

Comparisons of escape latency among control group, E1 group (pregnant rats exposure to enflurane for 4 h) and E2 group (pregnant rats exposure to enflurane for 8 h) when offspring rats were 20 and 30 days old respectively, (s,  $n=10$ ,  $\bar{x} \pm s$ ); a, vs, control group,  $P < 0.05$ .

**TABLE II** - Comparisons of numbers of offspring rats' crossing over the platform and times of offspring rats' staying in the target quadrant among Control group, E1 group and E2 group

Time	Group	Frequency of Crossing over the platform(No.)	Times of staying in the target quadrant(s)
Postnatal day 20	C group	2.9±0.7	30.0±3.2
	E <sub>1</sub> group	1.8±0.5 <sup>a</sup>	25.1±2.1 <sup>a</sup>
	E <sub>2</sub> group	1.8±0.5 <sup>a</sup>	26.6±2.4 <sup>a</sup>
Postnatal day 30	C group	2.5±0.7	29.3±3.5
	E <sub>1</sub> group	1.6±0.5 <sup>a</sup>	26.5±2.0 <sup>a</sup>
	E <sub>2</sub> group	1.6±0.5 <sup>a</sup>	26.1±2.2 <sup>a</sup>

Comparisons of numbers of offspring rats' crossing over the platform and times of offspring rats' staying in the target quadrant among Control group, E1 group (pregnant rats exposure to enflurane for 4 h) and E2 group (pregnant rats exposure to enflurane for 8 h) when offspring rats were 20 and 30 days old respectively, (s,  $n=10$ ,  $\bar{x} \pm s$ ); a, vs, control group,  $P < 0.05$ .

confirm that maternal exposure to enflurane anesthesia during early pregnancy could damage the spatial learning and memory in the resultant offspring and in a manner that correlates with NR2B mRNA expression in the hippocampus. NR2B mRNA expression levels in the E1 and E2 groups were not significantly different ( $P > 0.05$ ) from one another, which suggests that maternal enflurane anesthesia exposure during early pregnancy does not have an obvious time-dependent effect on offspring NR2B mRNA expression. These results are consistent with the Morris water maze test results. This further suggests that the damage caused by maternal enflurane anesthesia exposure in SD rats during early pregnancy impairs learning and memory in the offspring in a manner that is related to the inhibition of NR2B mRNA expression in the hippocampus (Table III).

### Immunohistochemistry assay

Table IV shows that at postnatal day 20, NR2B

**TABLE III** - Comparisons of the expression levels of NR2B mRNA in the hippocampus of offspring rats' postnatal day 20 and 30 among Control group, E1 group and E2 group

Time	Group	NR2B mRNA
Postnatal day 20	C group	0.71±0.07
	E <sub>1</sub> group	0.64±0.07 <sup>a</sup>
	E <sub>2</sub> group	0.63±0.07 <sup>a</sup>
Postnatal day 30	C group	0.67±0.04
	E <sub>1</sub> group	0.62±0.08 <sup>a</sup>
	E <sub>2</sub> group	0.58±0.05 <sup>a</sup>

Comparisons of the expression levels of NR2B mRNA in the hippocampus of offspring rats' postnatal day 20 and 30 among Control group, E1 group (pregnant rats exposure to enflurane for 4 h) and E2 group (pregnant rats exposure to enflurane for 8 h), (s,  $n=10$ ,  $\bar{x} \pm s$ ); a, vs, control group,  $P < 0.05$ .

protein levels in the hippocampus of rats from the E1 and E2 groups were  $148 \pm 28$  and  $128 \pm 17$ , respectively. These

levels were lower than those observed in the hippocampus of rats from the C group ( $162 \pm 17$ ) ( $P < 0.05$ ). At postnatal day 30, hippocampal NR2B protein levels in rats from the E1 and E2 groups were also significantly lower than in rats from the C group ( $P < 0.05$ ). This indicates that the enflurane-induced impairment of learning and memory is related to the down-regulation of NR2B protein expression in the hippocampus. NR2B protein expression levels between the E1 and E2 groups were not significantly different ( $P > 0.05$ ), which suggests that exposure to maternal enflurane anesthesia has no obvious time-dependent effect on NR2B protein expression, consistent with the Morris water maze test results.

**TABLE IV** - Comparisons of the expression levels of NR2B protein in the hippocampus of offspring rats' postnatal day 20 and 30 among Control group, E1 group and E2 group

Time	Group	NR2B protein
Postnatal day 20	C group	$162 \pm 17$
	E <sub>1</sub> group	$148 \pm 28^a$
	E <sub>2</sub> group	$128 \pm 17^a$
Postnatal day 30	C group	$154 \pm 12$
	E <sub>1</sub> group	$124 \pm 14^a$
	E <sub>2</sub> group	$119 \pm 15^a$

Comparisons of the expression levels of NR2B protein in the hippocampus of offspring rats' postnatal day 20 and 30 among Control group, E1 group (pregnant rats exposure to enflurane for 4 h) and E2 group (pregnant rats exposure to enflurane for 8 h), (s,  $n=10$ ,  $\bar{x} \pm s$ ); a, vs, control group,  $P < 0.05$ .

From the above results, we can conclude that maternal enflurane anesthesia exposure in SD rats during early pregnancy can impair spatial learning and memory in the resultant offspring. The mechanism of this process is related to the enflurane-induced inhibition of NR2B mRNA expression and NR2B protein levels in the hippocampus.

## DISCUSSION

Enflurane not only causes cognitive dysfunction in patients but also induces neurodegeneration, thereby posing a potential long-term risk to the central nervous system (Crosby *et al.*, 2010). The Morris water maze is a reliable means of assessing the cognitive capacity of animals (Feldman, Shapiro, Nalbantoglu, 2010) and was therefore used in this study to measure the cognitive capacity of offspring rats at postnatal days 20 and 30. Our results show that compared with rats in group C, those

in groups E1 and E2 had much longer escape latencies and lower numbers of crossings over the platform; they also spent less time in the target quadrant during the spatial exploration test. These results imply that maternal exposure to enflurane during early pregnancy can cause cognitive dysfunction in the resultant offspring.

Learning and memory occur when neurons undergo activity-dependent changes in synaptic strength in the mammalian central nervous system. Such synaptic plasticity could relate to changes in the connectivity underlying cognitive functions such as learning and memory (Kerrigan *et al.*, 2012). Synaptic plasticity mainly manifests as synaptic LTP and long-term depression (LTD), which are considered the biological foundation of learning and memory. LTD is related to information integration, forgetting, and memory recovery, whereas LTP is related to memory formation and storage. These two forms of plasticity constitute a neurophysiological network of learning and memory that is affected by many factors (Castillo, Chiu, Carroll, 2011; Lee, Kirkwood, 2011; Low, Wee, 2010; Pontrello *et al.*, 2012; Takagi *et al.*, 2012; Volianskis, Collingridge, Jensen, 2013; Zhu *et al.*, 2011). One such factor is the NMDAR, an important mediator that helps regulate synaptic plasticity and LTP (Pontrello *et al.*, 2012). Studies have found that the NMDAR has seven subunits (NR1, NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B) (Rammes *et al.*, 2011), with only NR2B showing high expression in hippocampal tissue. A positive correlation exists between NR2B expression and learning or memory capacity. Previous studies have demonstrated that NR2B up-regulates learning and memory competency by inducing LTP (Jung *et al.*, 2011; Rammes *et al.*, 2011; Yang *et al.*, 2011). Other studies have shown that down-regulation of NR2B expression in the brains of mammals impairs learning and memory (Brigman *et al.*, 2010; Farinelli *et al.*, 2012). Bliss (1999) referred to the NR2B gene as the "smart gene" or memory gene. Cao *et al.* (2007) also confirmed that transgenic rats with forebrain-specific over-expression of the NR2B subunit exhibit improved memory function and that mice lacking the NR2B gene (NR2B knockouts) exhibit lower memory function. By contrast, once the NR2B receptor is blocked by drugs, the behavior of rats is altered and LTP is inhibited in the hippocampus. These results demonstrate that NR2B is critical for learning and memory (Cui *et al.*, 2011; Fontán-Lozano *et al.*, 2011).

The present results show that the expression levels of NR2B mRNA and protein in the hippocampus of rats from the E1 and E2 groups are lower than that in rats from the C group. This indicates that the impairment of learning and memory caused by maternal enflurane

exposure during early pregnancy is mediated by the down-regulation of NR2B and the subsequent inhibition of LTP in the brain. Lin demonstrated that enflurane suppresses the oscillation and apparent desensitization of NMDA currents in oocytes, further inhibiting the generation of LTP (Lin, Chen, Harrid, 1993).

In conclusion, maternal enflurane exposure during early gestation could impair the cognitive function of resultant offspring. A possible mechanism for this action involves the down-regulation of NR2B expression.

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