The Impairment of Learning and Memory and Synaptic Loss in Mouse After Chronic Nitrite Exposure

Yongfang Chen,[1,2*] Zhanjun Cui,[1*] Lai Wang,[1] Hongliang Liu,[1] Wenjuan Fan,[1] Jinbo Deng,[1] Jiexin Deng[1]

[1]Institute of Neurobiology, School of Nursing, Henan University, Kaifeng 475004, People's Republic of China

[2]Luohe Medical College, Luohe 462002, People's Republic of China

Received 22 January 2015; revised 5 July 2015; accepted 6 July 2015

ABSTRACT: The objective of this study is to understand the impairment of learning and memory in mouse after chronic nitrite exposure. The animal model of nitrite exposure in mouse was created with the daily intubation of nitrite in adult healthy male mice for 3 months. Furthermore, the mouse's learning and memory abilities were tested with Morris water maze, and the expression of Synaptophysin and γ-Synuclein was visualized with immunocytochemistry and Western blot. Our results showed that nitrite exposure significantly prolonged the escape latency period (ELP) and decreased the values of the frequency across platform (FAP) as well as the accumulative time in target quadrant (ATITQ) compared to control, in dose-dependent manner. In addition, after nitrite exposure, synaptophysin (SYN) positive buttons in the visual cortex was reduced, in contrast the increase of γ-synuclein positive cells. The results above were supported by Western blot as well. We conclude that nitrite exposure could lead to a decline in mice's learning and memory. The overexpression of γ-synuclein contributed to the synaptic loss, which is most likely the cause of learning and memory impairment. © 2015 The Authors Environmental Toxicology Published by Wiley Periodicals, Inc. Environ Toxicol 31:1720–1730, 2016.

Keywords: learning and memory; nitrite exposure; synapse; γ-synuclein

INTRODUCTION

With regards to chemical properties, nitrite belongs to both strong oxidant and reducing agent, and the excessive intake of nitrite is harmful to heath. Nitrite exposure to humans comes mainly from water and foods. During food processing, especially canned foods, nitrite is a necessary additive to inhibit botox reproduction. Industrial wastewater and fertilizer are large source of nitrite as well. In addition, nitrite can also be produced from the decomposition of nitrate by bacteria in our body. Furthermore, some N-nitroso compounds can be combined with amines to generate nitrite. Therefore, nitrate, nitrite and N-nitroso compounds are considered as the harmful nitro substance. Nitrite acute poisoning is not uncommon, particularly with food intake. Although the toxicity of nitrite is known to people, previous studies mainly concentrated in the clinical treatment of acute nitrite poisoning (Schmidt and Clark, 2012; Yoo et al., 2013]. For chronic toxicity, the carcinogenic effects of nitrite have been given increased attention recently. Previous studies have showed that nitrite exposure was associated with the incidence of gastric cancer. Mirvish (1985) claimed that

*These authors contributed equally to this work.

Correspondence to: J.B. Deng; e-mail: jinbo_deng@henu.edu.cn or J.X. Deng; e-mail: dengjiexin@yahoo.com

Contract grant sponsor: National Natural Science Foundation of China.

Contract grant number: 31070952, U1204311.

Contract grant sponsor: Henan University Youth Training Fund.

Contract grant number: 0000A40356, 0000A40475.

Published online 27 July 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/tox.22174

the incidence of gastric cancer in Japan is six to eight times higher than in United States, since dietary intake of nitrite in Japan is three times higher than that in USA. Some studies have correlated nitrite intake with injuries in central nervous system (CNS), for instance, nitrite exposure during pregnancy could probably lead to fetal neural malformations (Brender et al., 2004). Compared with the studies on acute poisoning and canceration, CNS injuries after chronic nitrite exposure is not fully understood, particularly with regards to learning and memory impairment.

Whether nitrite exposure can cause learning and memory impairment in animals is extremely important to gauge its risk to intellectual development of school children. It was reported that prenatal sodium nitrite exposure could evoke a decrease in the birth and survival rates of pups and lead to a disturbance in the cognitive ability (mainly learning and memory) in pregnant mice (Koziar et al., 1994). Hemic hypoxia probably contributes to cognitive disorder, since nitrite is believed to be a methemoglobin-inducing agent (Koziar et al., 1994). However, synapse is considered to participate in the processes of learning and memory as an important structure in neural network. No doubt, alterations in synaptic quantity and structure will affect learning and memory.

To understand the learning and memory disorder and synaptic loss after nitrite exposure, a nitrite exposure model was established in mice, and their learning and memory were tested with water maze. Synaptophysin (SYN) is regarded as a synaptic relative protein in presynaptic element, and it is often used as a specific marker for synapses in central nervous system (Gordon and Cousin, 2014). The amount of synaptophysin positive buttons may roughly represent the relative quantity of synapses (Sherwood et al., 2010). In the study, the synaptophysin positive buttons in visual cortex were visualized and quantified, with or without nitrite exposure. As a regulator of neurotransmitter and synaptogenesis, synuclein is also analyzed, especially the correlation between synaptophysin and γ-synuclein expression. The present study will be helpful for us to evaluate the hazard of nitrite exposure and to inform regulatory decisions on the food and water quality standards.

MATERIALS AND METHODS

Animals and Grouping

All experiments were carried out in accordance with institutional guidelines of Henan University for animal welfare. Adult male C57BL/6J mice (6- to 8-week old) were fed in standard laboratory animal housing with 12 h: 12 h light:-dark cycle, at 20–25°C and 50–60% humidity. The 73 male mice were randomly divided into two groups, control group (control, C) and nitrite treatment groups. The following was the details of animal grouping: ① Nitrite treatment groups: Males received daily intragastric gavages of 6 g L^{-1} nitrite at a dose of either 0.06 g kg^{-1} (moderate dose) or 0.12 g kg^{-1} (high dose) for 3 months (Hu et al., 2015; Zhang et al., 2015). To allow the stomach to empty and to facilitate the absorption of nitrite, food was removed 2 h before nitrite dosing. Animals were weighed and gavaged at the same time each day. ② Control group: Males were intubated with same quantity of saline instead of nitrite. At least 18 nitrite-exposed mice in each group were used for the continuous data collection. More than six mice in each group were used for water maze tests, and at least five mice in each group were used for immunochemistry, with at least five mice for Western blot analysis.

Behavior Assays with Water Maze

Morris water maze can be used to test mice' ability of learning and memory, through searching for spatial positions (platform). Morris maze (MT-200, Taimeng Biotechnology, Chengdu, China) was used for the experiment. The pool was 100 cm in diameter and 50 cm in height. Water (25–28°C), dyed with the same milky color as maze wall, was filled into pool by 1–2 cm above platform. The swimming pool was divided fictitiously into four quadrants, and the platform was located in the quadrant 2. The positioning navigation (acquired training) and spatial exploration assays were carried out to understand mouse's learning and memory respectively. For positioning navigation assay, mice dived into water from quadrant 4 near the pool's wall and then started to swim and search for the target platform. By chance, the swimming mice would meet the platform and stay here. The staying over 3 s were regarded as success. Escape latency period (ELP), the swimming time from diving to reaching platform, was recorded. If the mice failed to find the platform over 60 s, then, they would be guided to reach the platform and stay there for about 10 s as training. For these mice, 60 s were defined as their escape latency period. The escape latency period would be tested for consecutive 7 days with four repetitions per day, and the interval time between two near assays was more than 30 min. At day 8, the platform in water was removed, and the mice were used for further space exploration assay. After diving into water, the swimming mice were still trying to find target platform, even though platform no more existed. During searching time for 60 s, the frequency across platform (FAP) and accumulative time in target quadrant (ATITQ) were recorded, in order to detect the mouse's space memory ability (Vorhees and Williams, 2014).

Immunofluorescent Labeling

Mice were anesthetized with sodium pentobarbital (20 mg kg^{-1}, i.p.) and perfused transcardially with 4% paraformaldehyde (PFA) in 0.01 M phosphate buffer (PB) (pH 7.2). Then, whole brains were removed, and fixation was continued at 4°C for 1–2 days. In this study, the expression of

synaptophysin and γ-synuclein were observed with immunofluorescent labeling so that the number of synapses (synaptophysin positive puncta) and their regulatory molecule (γ-synuclein) after nitrite exposure could be investigated (Masliah et al., 1992). Because murine cortex is laminated with six layers, layers V–VI were used as our target in the study to label the synaptophysin positive puncta and γ-synuclein positive neurons. CDP (specific protein for the neurons of layer II) and FOXP2 (specific protein for layer V-VI) were used to locate layer II–VI. The visual cortex was sectioned coronally (50 μm) using a vibratome. Sections were rinsed in 0.01M PB and preincubated in blocking solution (5% normal goat serum) for 30 min at room temperature before immunofluorescent labeling. Then sections were incubated overnight at 4°C with the indicated dilutions of primary antibodies: polyclonal rabbit anti-FOXP2 antibody (1: 600, Abcam, AB16046), goat anti-CDP polyclonal antibody (1: 400, Santa Cruz, SC6327), rabbit anti-synaptophysin polyclonal antibody (1: 300, Millipore, AB9272), rabbit anti-gamma synuclein polyclonal antibody (1: 500, Abcam, AB55424). After multiple washes in 0.01 M PB, appropriate secondary antibodies were added at the indicated dilutions and incubated at room temperature for 3 h. The secondary antibodies were: Alexa Fluro 488 donkey anti-rabbit IgG (1:300; Invitrogen, A21206) and Alexa Fluro 568 goat anti-rabbit IgG (1:600; Invitrogen, A11057). Sections were counterstained with DAPI (4′, 6-diamidino-2-phenylindole, 1:5000; Santa Cruz, SC3598) for 1–2 min after immunolabeling. Finally, sections were coverslipped with 65% glycerol in 0.01 M PB and imaged using an epifluorescence microscope (BX61, Olympus, Japan) with rhodamine, fluorescein isothiocyanate (FITC) or ultraviolet filter sets. High-quality sections were photographed using an Olympus laser confocal microscope (FV1000, Olympus, Japan), using separate scans with 568 nm (red) and 488 nm (green) laser lines.

DiI Diolistic Assay

DiI(1, 1′-dioctadecy 1-3,3,3′-tetramethylindocar bocyanine perchlorate) diolistic assay can be used to visualize neuronal dendritic spines. After fixation, coronal sections (50 μm) were cut using a vibratome. Slices were washed and stored in 0.01 M phosphate buffer (PB) before use. Gene Gun bullets were prepared by a modified method (Deng et al., 2006). After the bullets with DiI-coated gold were prepared, DiI delivery could be carried out. Brain slices were transferred to a Petri dish with 0.01M PB (pH 7.2). After PB was drained, DiI-coated particles were delivered using the Helios Gene Gun system (Bio-Rad, Hercules, CA) at a pressure of 150 psi. After delivery, slices were incubated in 0.01M PB at 4°C overnight to allow for diffusion of the dye along the neuronal processes. After being washed three times, sections were counterstained with DAPI (1:5000, Santa Cruz, SC3598) and coverslipped with 65% glycerol in 0.01 M PB. Finally, the sections were photographed under a laser confocal microscope (FV1000; Olympus, Japan) with separate scanning at 356 nm (for DAPI) or 568 nm (for DiI) laser wavelength.

Transmission Electron Microscopy

Anesthetized mice (sodium pentobarbital, 20 mg kg^{-1}, i.p.) were perfused transcardially with 4% paraformaldehyde and 1% glutaraldehyde in 0.01 M PB. Visual cortices were dissected into tissue blocks (1 mm × 1 mm × 1.5 mm) using a razor and postfixed in 4% glutaraldehyde for 4–12 h at 4°C. After washing for several times in PB, all samples were postfixed in 1% OsO4 for 1 h before dehydration in graded EtOH and embedding in Epon 812 resin (Durcupan ACM; Sigma–Aldrich, Gillingham, United Kingdom). Layers V–VI were localized in semi-ultrathin sections under light microscopy, and ultrathin 70–80 nm sections were cut (Reichert Ultracut E; Leica, Austria) and stained with uranyl acetate, followed by lead citrate. The synapses in layers V–VI were imaged with a Hitachi H-7500 electron microscope.

Western Blot Assay

Western blot was used further to test the expression of synaptophysin in visual cortex after nitrite exposure. Mice were sacrificed by cervical dislocation. After opening the skull, brains were quickly taken away. After first separating visual cortices for homogenization, cortical proteins were extracted using a protein extraction kit (Shanghai Beyotime Institute of Biotechnology, Shanghai, China, P0027). The proteins were quantified using the Coomassie brilliant blue method. Sample protein concentration in different groups was calculated with standard curves. The proteins were electrophorized and subsequently electro-transferred onto PVDF membranes. Rabbit anti-Synaptophysin polyclonal antibody (1: 5000; Millipore, AB 9272) was added to the membrane and incubated overnight at 4°C. After washing with PBS for three times, horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2000; Beijing Dingguo Changsheng Biotechnology) was then added to the membrane for 2-h incubation at room temperature. After enhanced chemiluminescence reagent was added onto membrane, exposure and development of film were carried out. β-actin, detected with mouse anti monoclonal antibody (1:1000; Shanghai Beyotime Institute of Biotechnology, Shanghai, China, AA128), was used as the internal control. The optical absorption ratio of synaptophysin bands to internal control (β-actin) bands represented the relative expression of synaptophysin.

Statistical Analysis

Measurements

The test of escape latency period (ELP) was carried out from day 1 to 7 to indicate the learning ability of mice in various

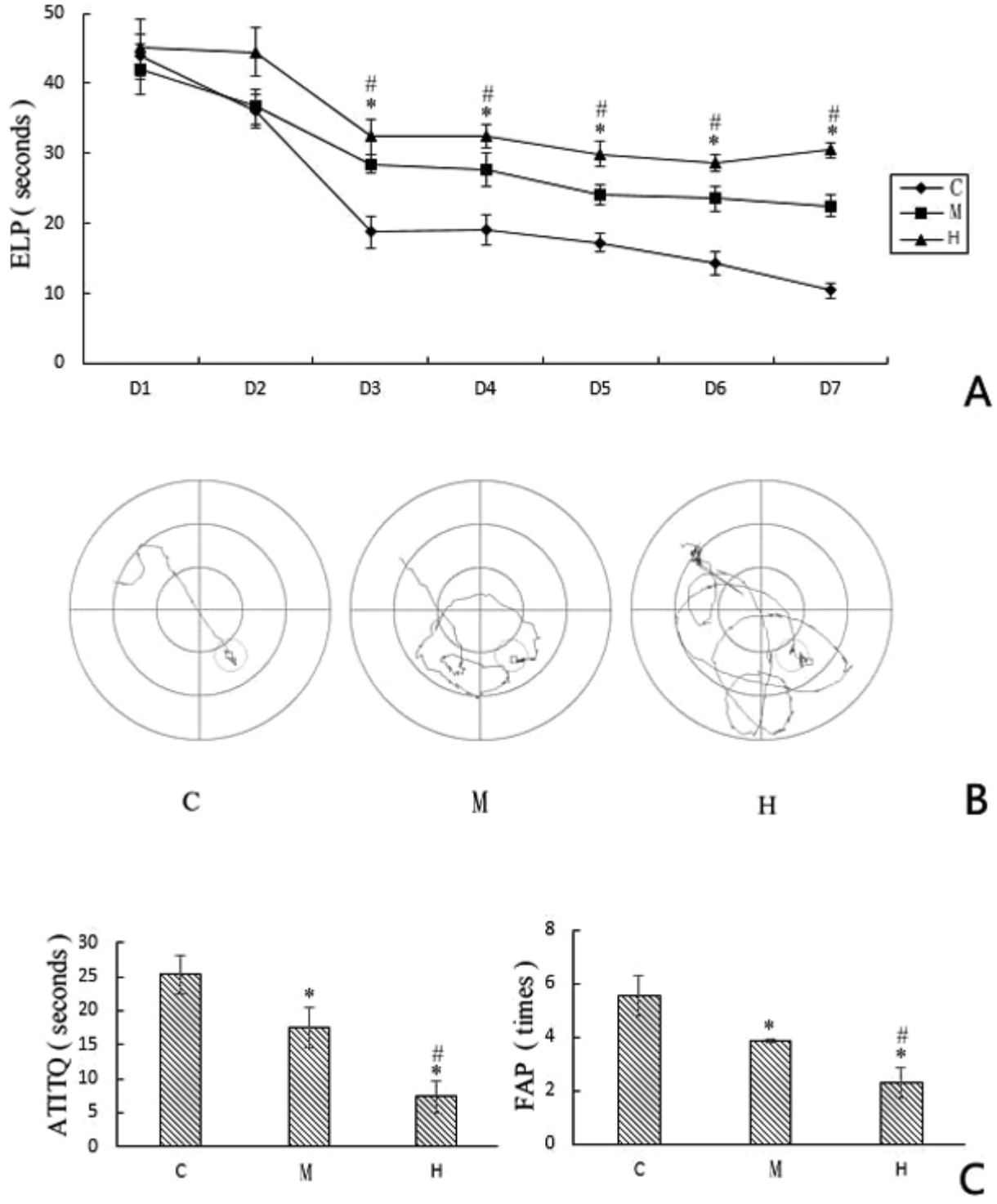

Fig. 1. Learning and memory of mice after nitrite exposure. A: Escape latency period (ELP) was measured in various groups at different time. During the first 2 days, there was no difference among various groups ($P > 0.05$). After day 3, ELP was longer in nitrite exposure groups compared to control with dose dependency ($P < 0.05$), and from day 7 onward, the differentiation became more significant ($P < 0.01$). B shows the orbits of platform seeking in various groups at day 7. C: the frequency of target quadrant search (FAP) and the accumulative time in target quadrant (ATITQ) decreased after nitrate exposure with dose dependency ($P < 0.05$). *: $P < 0.05$ for nitrate treatments versus control; #: $P < 0.05$ for high- versus moderate-dose treatment. Control, moderate and high dose treatment are marked with C, M, and H, respectively.

groups. At day 8, the frequency across platform (FAP) and accumulative time in target quadrant (ATITQ) were tested as well. These parameters reflected the memory ability of mice in various groups. Image J software analysis was used to measure the synaptophysin positive puncta, γ-synuclein positive neurons in layer V–VI. Western blot semi-quantitative analysis was used to semi-quantify the synaptophysin expression. The following is the measurements: ① ELP = the time for mouse to find the platform (seconds); ② FAP = the frequency across platform in 60s; ③ ATITQ = the accumulative time in target quadrant in 60s; ④ Density of synaptophysin positive puncta = the number synaptophysin positive puncta/cortical area (puncta/μm^2); ⑤ Density of γ-synuclein positive neuron = the number of γ-synuclein positive cells/cortical area (cells/mm^2); ⑥ Synaptophysin expression = SYN gray value/β-actin gray value; ⑦ Density of spines (DS): DS = number of spines/length of dendrites; ⑧ Average length of spines (ALS): ALS = total length of spines/number of spines.

Statistical Analysis

More than five mice were used in every measurement. In addition, for immunofluorescent staining, at least five sections were prepared for each mouse. All data were calculated as mean value ± standard deviation ($\overline{x} \pm s$). With Spss11.5 statistical analysis software, ANOVA analysis was carried out to compare the difference among various groups. $P < 0.05$ was accepted as statistical significance.

RESULTS

Effects of Nitrite Exposure on Learning and Memory

In nitrite treatment groups, the mice became small in size, listless in spirit, slow in action and so on. In the present study, six mice in each group participated in water maze assay. The factor of physical strength was ruled out since there was no significant difference in swimming speed between control and nitrite exposure groups ($P > 0.05$). In first 2 days, there was no statistical difference among various groups ($P > 0.05$), although ELP was relatively long for every group. With more learning, the time for mice to find platform was shortened, and difference started to appear among groups after day 3. ELP was longer in nitrite treatment groups than in control, in a dose-dependent manner [$P < 0.05$, Fig. 1(A)]. By examining the recorded swimming orbits at day 7, the trajectory in the control group was found to be shortest in all groups, while the orbit in high-dose group was the longest and most complex in all groups [Fig. 1(B)]. At day 8, mice's ability of space exploration (FAP and ATITQ assays) was compared among various groups. Both FAP and ATITQ were reduced in treatment groups in a dose-dependent manner [$P < 0.05$ dose Fig. 1(C)]. These phenomena suggest that nitrite exposure could reduce learning and memory with dose dependency.

Nitrite Exposure and Synaptic Loss and Damage

Synaptophysin (SYN) immunofluorescent staining was performed in order to investigate the mechanism of alterations of learning and memory after nitrite exposure. Although hippocampus is very important organ for learning and memory, however, SYN's expression was too dense and strong to identify a single SYN positive button. Therefore, an accurate count was impossible. On the other hand, visual cortex carried out "higher brain functions" including stimulus familiarity, reward-timing prediction and spatiotemporal sequence learning (Gavornik and Bear, 2014). Therefore, the visual cortex was targeted in the study as our previous studies on alcohol toxicology (Cui et al., 2010). Because CDP and FOXP2 were specific neuronal markers in layer II–III and layer V–VI of neocortex (Rowell et al., 2010), FOXP2 and CDP immunolabeling was also used to visualize the lamination of visual cortex. The target area was focused on layer V–VI in which the neurons were Foxp2 positive [Fig. 2(A)]. Figure 2(B) shows the expression of synaptophysin in visual cortex. The synaptophysin positive puncta was mainly located in the surface and processes of neurons, and their fluorescent puncta could be used to quantify the synapses in cortex roughly. Figure 2(C–E) shows the high magnification of synaptophysin positive puncta in various groups. The cell bodies (blank areas without puncta) were surrounded by synaptophysin positive puncta. In our study, nitrite exposure could cause synaptic loss in a dose-dependent manner. To support our observation statistically, we measured synaptophysin positive puncta per unit area for statistical testing among various groups. There were significant differences among various groups [$P < 0.01$, Fig. 3(A)], suggesting that nitrite exposure could reduce the number of synapses with dose dependency. Western blot semi-quantitative analysis also supported our results from synaptophysin immunochemistry [Fig. 4(A)]. The relative expression of SYN (SYN optical density/β-actin optical density) in visual cortices was analyzed in various groups. There were significant dose-dependent differences among the various groups [$P < 0.01$, Fig. 4(B)].

Excitatory synapses are generally located on the surface of dendritic spines; therefore dendritic spines can be used as an indirect indicator of synaptic alterations. After nitrite exposure, there were quantitative differences between the treatment groups and controls. The numbers of dendritic spines of pyramidal cells in layer V–VI were reduced in the nitrite exposure groups compared to controls [Fig. 2(F–H)]. This finding suggested that nitrite exposure reduced dendritic spine density in pyramidal cells. At the same time, the overall length of the spines was significantly increased in exposed animals. In Figure 5, the histograms represent

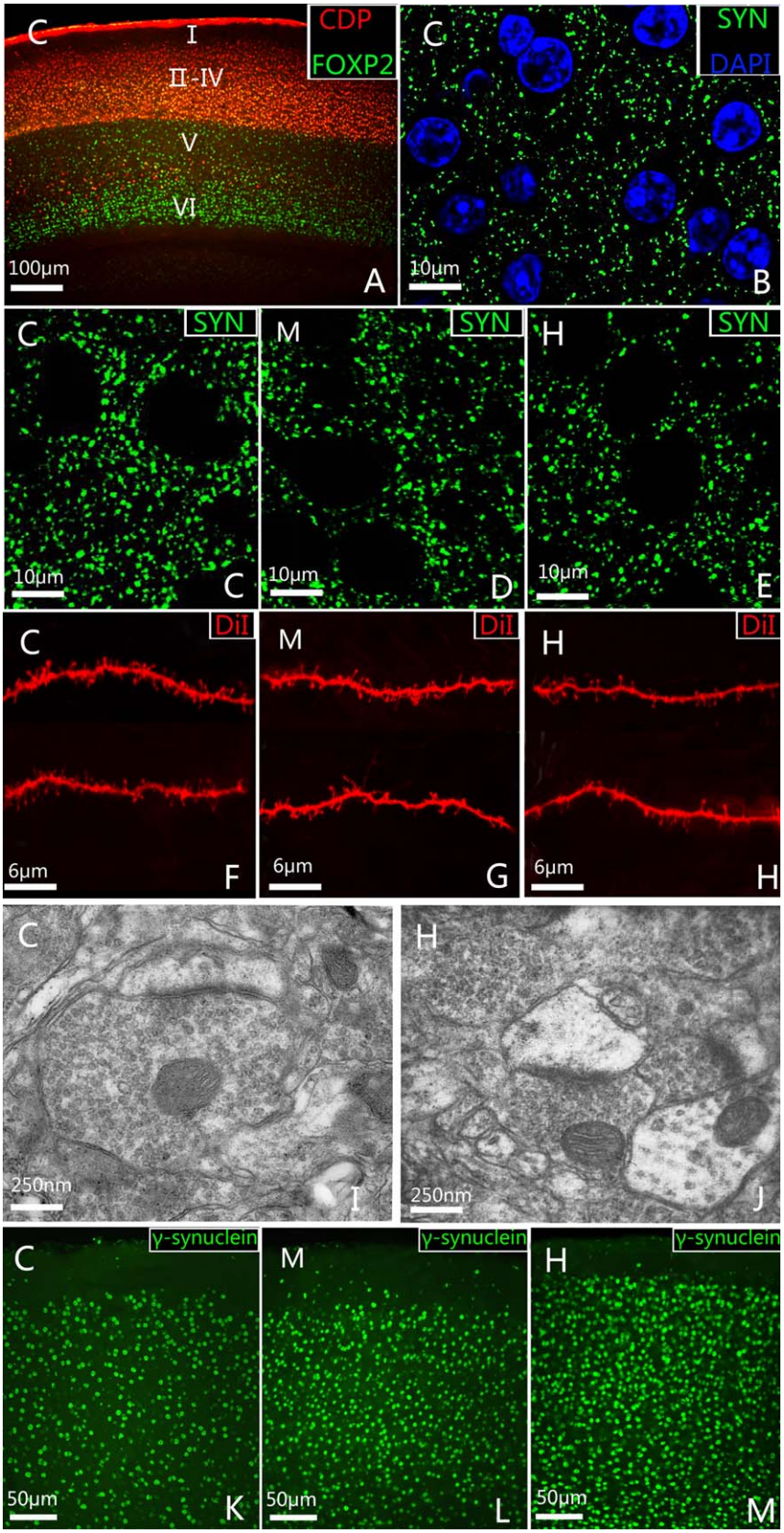
C
I
II-IV
V
VI
CDP
FOXP2
100μm
A
C
SYN
DAPI
10μm
B
C
SYN
10μm
C
M
SYN
10μm
D
H
SYN
10μm
E
C
DiI
6μm
F
M
DiI
6μm
G
H
DiI
6μm
H
C
250nm
I
H
250nm
J
C
γ-synuclein
50μm
K
M
γ-synuclein
50μm
L
H
γ-synuclein
50μm
M

Fig. 2.

measurements of density of spine (DS) and average spine lengths (ALS) as well as statistical comparisons among the various groups. After nitrite exposure, DS values were reduced, but ALS values were increased in a dose-dependent manner.

Nitrite exposure could also induce synaptic alterations at the ultrastructural level. In the control group, synapses showed normal ultrastructure, in which the presynaptic element, synaptic cleft, and postsynaptic element could be recognized [Fig. 2(I)]. The presynaptic element contained many presynaptic vesicles and mitochondria, and there was a gap of 25–30 nm for the synaptic cleft. The postsynaptic membrane thickening and specialization were prominent in the postsynaptic element [Fig. 2(I)]. After nitrite exposure, some organelles in presynaptic elements and postsynaptic elements were degraded. Some microtubules in postsynaptic density had started to disintegrate, and the presynaptic and postsynaptic membranes became fused, with the synaptic cleft being degenerated into a vague structure [Fig. 2(J)].

Nitrite Exposure and γ-Synuclein Expression

Generally, Synuclein is associated with the familial neurodegenerative diseases, particularly Parkinson's disease (PD) (Surguchov, 2013). Recently, synuclein has also reported to serve regulatory functions of neurotransmitter release and synaptogenesis (Vargas et al., 2014). In this study, we tried to understand whether γ-synuclein was correlated with synaptotic loss after nitrite exposure. We found that γ-synuclein were mainly expressed in neuronal nuclei of visual cortex, and the density of γ-synuclein positive cells in layer V–VI of visual cortex was increased after nitrite exposure in a dose-dependent manner [Fig. 2(K–M)] [$P < 0.05$, Fig. 3(B)]. In the meantime, in order to understand the causality between synaptophysin and γ-synuclein, a correlation test was made between synaptophysin positive puncta and γ-synuclein positive cells. There was a negative correlation between them ($r = 0.881$, $P < 0.01$), suggesting that γ-synuclein down-regulate the number of synapses in visual cortex.

DISCUSSION

Food safety has always been the focus of concern to people. Nitrite is often added in the canned foods for preservation, therefore nitrite exposure for people is not avoidable. Another way for nitrite exposure is from drinking water, since industrial pollution (such as paper mill) and fertilizer application are considered to be a main water source of nitrite exposure especially in developing countries. The biological effects of nitrite are mutifaceted. Its metabolite, such as NO, even is an important secondary messenger which is involved in essential cellular activities (Shiva et al., 2007). However, the toxicity of nitrite should not be neglected. Tens of thousands of people have died due to acute nitrite toxicity every year in China. Nitrite chronic toxicity is also dangerous for people, since it can be associated with carcinogenicity and teratogenicity (Scragg et al., 1982; Wang et al., 2012; Toya et al., 2012). Base on previous studies, high nitrate in drinking water may be associated with a high incidence of congenital malformations (Winchecter et al., 2009). In China, Li et al. (2006) reported that if pregnant mothers were in the habit of eating pickles, their children would have a higher incidence of neural tube defects, since pickles contained high amounts of nitrite. Nevertheless, the CNS injuries after nitrite exposure, particularly cognitive impairment and synaptic loss, are not fully understood well. In this study, the impairment of learning and memory and synaptic loss after chronic nitrite exposure was investigated, and certainly our study will provide some new knowledge about nitrite exposure.

Nitrite Exposure Impairing Learning and Memory

Learning and memory are essential functions of CNS. With Morris water maze, alterations in learning and memory after nitrite exposure have been tested in mice. In the present study, the parameters, such as escape latency period (ELP), frequency across platform (FAP) and accumulative time in

Fig. 2. Synapses and γ-synuclein positive cells in visual cortex (immunolabeling). Photo A shows the lamination of cortex with CDP (red) and FOXP2 immunolabeling. CDP was mainly expressed in layer II–IV, and FOXP2 was expressed in layer V and VI which was selected as target area. Photo B shows the high magnification of presynaptic buttons (green, synaptophysin immunolabeling) with DAPI counterstaining (blue). After nitrate exposure, the Synaptophysin positive buttons decreased with dose dependency (C–E). In the meantime, the dendritic spines were also changed after nitrite exposure, and nitrite exposure could cause dendrites to appear sparser and longer with dose dependency (F–H). I–J: Changes in synaptic ultrastructure following nitrite exposure. The typical synaptic ultrastructure with normal presynaptic membrane, synaptic cleft and postsynaptic membrane can be seen in control (I). After high dose exposure, dark and thick postsynaptic density appears prominently in the postsynaptic membrane, accompanied by a narrowing of the synaptic cleft (J). On the other hand, the γ-Synuclein positive cells increased after nitrite treatment (K–M). Control, moderate- and high-dose treatment are marked with C, M, and H, respectively.

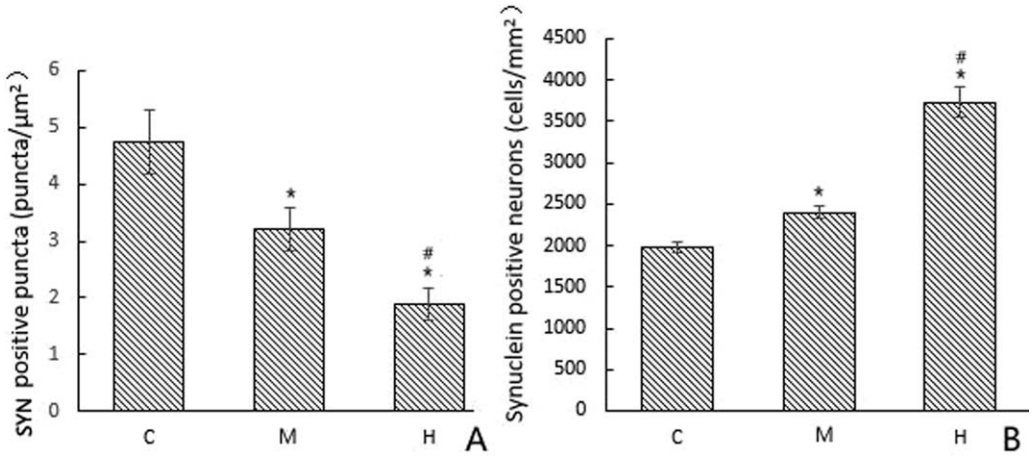

Fig. 3. Histogram and statistical analysis of synaptophysin positive buttons and γ-synuclein positive cells. Nitrite exposure can induce the loss of presynaptic buttons ($P < 0.01$, A) and the increase of γ-synuclein positive cells with dose dependency ($P < 0.05$, B). Columns are the mean values ± standard deviation (SD). Statistical analysis was performed with one-way analysis of variance (ANOVA) q tests. ∗: $P < 0.05$, if nitrite treatments versus control; #: $P < 0.05$, if high- versus moderate-dose treatment. Control, moderate- and high-dose treatment are marked with C, M, and H, respectively.

target quadrant (ATITQ), were used to test the impact of nitrite exposure on learning and memory (Liu et al., 2013). Initially, the learning ability of animals was studied with acquired training. After nitrite exposure, ELP was much longer with more complicated swimming orbits compared to control, in a dose-dependent manner. On the other hand, the ability of space exploration was tested with FAP and ATITQ assays in order to understand the memory ability of mice. At day 8, the FAP and ATITQ of animals also had significant differences among various groups, and nitrite exposure could reduce the values of FAP and ATITQ with dose dependency. These phenomena suggested that the learning and memory of mice were impaired after nitrite exposure with dose-dependency. It is extremely important for us to avoid nitrite exposure as much as possible, especially during the period of intellect development for the school teenagers. On the other hand, it also implies us the possible correlation between Alzheimer's disease and nitrite exposure. Some studies have showed that microglia could release of nitric oxide to cause the action of beta-amyloid which is a specific pathological property of Alzheimer's disease (Goodwin et al., 1995, 1997).

Synaptic Alterations Being the Cause of Learning and Memory's Impairment

The biological basis of learning and memory is very close to synaptic functions among CNS networks. Generally, the limbic system, particularly hippocampus, is believed to be the organs to participate in learning and memory. However, cerebral cortex is probably involved in the processes of learning and memory as well. Electroencephalogram (EEG) data show that learning and memory requires the participation of numerous subcortical and cortical neurons (Hamm et al., 1992; Gevins et al., 1997). In addition, in AD patients, the amount of cholinergic neurons in neocortex is associated with learning and memory abilities (Li et al., 2012). In this way, visual cortex is a suitable target to analyze the alteration of synapses after nitrite exposure.

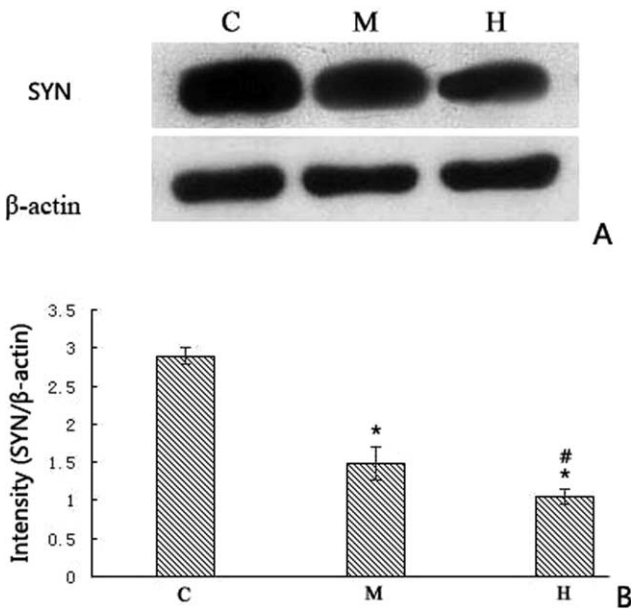

Fig. 4. Synaptophysin expression in visual cortex by Western blot. A: the expression bands of Synaptophysin in various groups, and β-actin was used as internal control. B: Semi-quantitative analysis of Synaptophysin was made in various groups. ∗$P < 0.05$, if nitrite treatments versus control. #: $P < 0.05$, if high- versus moderate-dose treatment. Control, moderate- and high-dose treatment are marked with C, M, and H, respectively.

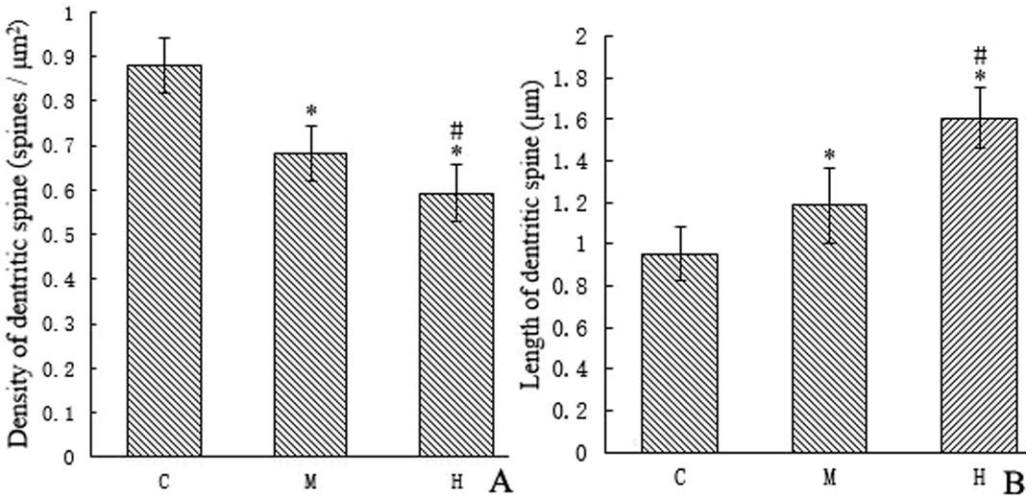

Fig. 5. Dendritic spine length and spine density following nitrite exposure. A: Nitrite exposure could lead to a reduction in dendritic spine density with dose dependency. B: Nitrite exposure induces the elongation of dendritic spines with dose dependency. Columns are the mean values ± standard deviation (SD). Statistical analysis was made with one-way analysis of variance (ANOVA) *q* tests. $*P < 0.05$, if nitrate treatments versus control. #: $P < 0.05$, if high- versus moderate-dose treatment. Control, moderate- and high-dose treatment are marked with C, M, and H, respectively.

To elucidate the mechanisms of learning and memory impairment after nitrite exposure, synaptic pathology, such as synaptic density, dendritic spines and synaptic ultrastructure were evaluated comprehensively. Synaptophysin can be used as a marker of presynaptic terminals. With immunofluorescent labeling and Western blot analysis, we found that synaptophysin positive puncta and its expression in visual cortex were decreased after nitrite exposure. In addition, the dendritic spines and synaptic ultrostructure were analyzed with DiI diolistic assay and transmission electron microscopy. We found that nitrite exposure also made dentritic spine appear sparser and longer than control group in a dose-dependent manner. Under electron microscope, nitrite exposure could cause a thickening of presynaptic membrane and postsynaptic membrane. In treatment groups, synaptic cleft became narrow, and presynaptic density and postsynaptic density started to fuse with each other. All the changes above pointed to the conclusion that nitrite exposure could induce synaptic loss and structural damage. The synaptic loss and damage probably are the main causes of learning and memory impairment after nitrite exposure. As we know that many biochemical reactions associated with learning and memory occur in synapses. The activity of AMPA receptor and NMDA in synapses often follows the processes of learning and memory. Once the learning and memory start, Ca^{++} will influx into cytoplasm, and calcium-calmodulin-dependent protein kinase II (CaMK II) and protein kinase C (PKC) are activated. With serial signal cascade, AMPA receptors are phosphorylated, and the memory is produced (Tinsley et al., 2009). Synaptic abnormality can also cause some neurological and psychiatric disorders such as schizophrenia (Faludi and Mirnics, 2011; Karlsen et al., 2013) and Alzheimer's disease (AD) (Calkins and Reddy, 2011). In these diseases, the number of SYN positive puncta in visual cortex was significantly reduced. Coincidentally, these diseases not only display learning and memory problems but also synaptic loss and damage, suggesting that synapse may play an important role in animals' cognitive ability. In addition, as a marker of presynaptic element, SYN is also involved in the cellular functions, such as synaptogenesis, Ca^{++} dependent neurotransmitter transfer and long-term potential (LTP), which are related to learning and memory (Bliss and Lømo, 1973). Down-regulation of SYN itself will reduce the ability of learning and memory directly.

Synuclein Regulating the Synaptic Activity

Synuclein family including α-synuclein (SNCA), β-synuclein (SNCB), and γ-synuclein (SNCC) exists in the central nervous system widely and is expressed in the nuclei and presynaptic elements. The protein family is highly homologous in their amino acid sequence and their spatial structure. Synuclein has wide physiological functions and pathological significance. Synuclein's abnormal expression is closely related to tumorigenesis, such as breast cancer, ovarian cancer, Kaposi sarcoma and some malignant hemangioma (Zhang et al., 2011). Synuclein is also strongly associated with some degenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease. The main physiological function of Synucleins is to regulate the kinetics of synaptic vesicle endocytosis (Vvargas et al., 2014) and synaptogenesis (Zhong et al., 2010). Under the participation of calcium ions, Synuclein can trigger the fusion of synaptic vesicles with presynaptic membrane to release neurotransmitter. Without

synuclein activity, synaptic vesicles would accumulate in presynaptic element, and the neurotransmitter cannot be released properly (Bankiewicz et al., 1990). Therefore, maintaining synuclein's normal structure and quantity is very important for nervous activity (Buchman et al., 1998). In fact, both Parkinson's disease (PD) and Alzheimer's disease (AD) are often associated with abnormal release of neurotransmitter and the synaptic loss and damage, which could be due to involvement by synucleins. For instance, scientists found that in both PD and AD, Synuclein oligomers often accumulate at the pre-synaptic membrane and trigger synapse degeneration (Kramer and Schulz-Schaeffer, 2007; Kazantsev and Kolchinsky, 2008). Therefore, the accumulation of synuclein can cause synaptic damage or loss (Scott et al., 2010). In this study, we have investigated the expression of synaptophysin and γ-synuclein in visual cortex with immunolabeling and tested the correlation between them as well. We found that after nitrite exposure, γ-synuclein positive cells were increased in a dose-dependent manner, suggesting an abnormal accumulation or overexpression of γ-synuclein after nitrite exposure. The overexpression of γ-synuclein was followed by synaptic reduction, suggesting that the accumulation of synuclein could contribute to the loss of synapses after nitrite exposure, probably due to the toxicity of synuclein to synapses. Synuclein's toxicity mainly comes from insoluble synuclein rather than soluble synuclein. Under nitrite stimulation, insoluble synuclein would increase quickly. These accumulated insoluble synuclein in presynaptic element prevented the normal functioning of neurotransmitter release, resulting in synaptic loss and learning and memory impairment (Su et al., 2003).

In summary, nitrite exposure could induce learning and memory impairment in mice. Synaptic loss and damage in cortex probably contributes to learning and memory reduction. Abnormal accumulation of γ-synuclein and its toxicity are involved in synaptic loss in cortex. Our study provides some useful data for health authorities to figure out food and water quality standards.

REFERENCES

Bankiewicz KS, Plunkett RJ, Jacobowitz DM, Porrino L, di Porzio U, London WT, Kopin IJ, Oldfield EH. 1990. The effect of fetal mesencephalon implants on primate MPTP-induced parkinsonism: Histochemical and behavioral studies. J Neurosurg 72:231–244.

Bliss TVP, Lømo T. 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol 232:331–356.

Brender JD, Olive JM, Felkner M, Suarez L, Marckwardt W, Hendricks KA. 2004. Dietary nitrites and nitrates, nitrosatable drugs, and neural tube defects. Epidemiology 15:330–336.

Buchman VL, Hunter HJ, Pinõn LG, Thompson J, Privalova EM, Ninkina NN, Davies AM. 1998. Persyn, a member of the synuclein family, has a distinct pattern of expression in the developing nervous system. J Neurosci 18:9335–9341.

Calkins MJ, Reddy PH. 2011. Amyloid beta impairs mitochondrial anterograde transport and degenerates synapses in Alzheimer's disease neurons. Biochim Biophys Acta 1812:507–513.

Cui ZJ, Zhao KB, Zhao HJ, Yu DM, Niu YL, Zhang JS, Deng JB. 2010. Prenatal alcohol exposure induces long-term changes in dendritic spines and synapses in the mouse visual cortex. Alcohol Alcohol 45:312–319.

Deng J, Yu D, Wu P. Introduction of DiI diolistic assay to label the nervous cell and glia. Acta Anatom Sin 2006;37:596–598.

Faludi G, Mirnics K. 2011. Synaptic changes in the brain of subjects with schizophrenia. Int J Dev Neurosci 29:305–309.

Gavornik JP, Bear MF. 2014. Higher brain functions served by the lowly rodent primary visual cortex. Learn Mem 21:527–533.

Gevins A, Smith ME, McEvoy L, Yu D. 1997. High-resolution EEG mapping of cortical activation related to working memory: Effects of task difficulty, type of processing, and practice. Cereb Cortex 7:374–385.

Goodwin JL, Uemura E, Cunnick JE. 1995. Microglial release of nitric oxide by the synergistic action of β-amyloid and IFN-γ. Brain Res 692:207–214.

Goodwin JL, Kehrli ME Jr, Uemura E. 1997. Integrin Mac-1 and beta-amyloid in microglial release of nitric oxide. Brain Res 768:279–286.

Gordon SL, Cousin MA. 2014. The Sybtraps: Control of synaptobrevin traffic by synaptophysin, α-synuclein and AP-180. Traffic 15:245–254.

Hamm RJ, Dixon CE, Gbadebo DM, Singha AK, Jenkins LW, Lyeth BG, Hayes RL. 1992. Cognitive deficits following traumatic brain injury produced by controlled cortical impact. J Neurotrauma 9:11–20.

Hu S, Cui ZJ, Guo JN, Yan MC, Deng JB, Deng JX. 2015. Effects of prenatal nitrite exposure on hippocampus of mouse pups. Acta Anatom Sin 46:289–296.

Karlsen AS, Kaalund SS, Møller M, Plath N, Pakkenberg B. 2013. Expression of presynaptic markers in a neurodevelopmental animal model with relevance to schizophrenia. Neuroreport 24: 928–933.

Kazantsev AG, Kolchinsky AM. 2008. Central role of α-synuclein oligomers in neurodegeneration in Parkinson disease. Arch Neurol 65:1577–1581.

Koziar VS, Trofimov SS, Ostrovskaia RU, Sariev AK, Zherdev VP. 1994. Prenatal exposure to sodium oxybutyrate prevents a disorder of general behavior, learning and memory in the progeny of rats subjected to chronic hemic hypoxia. Eksp Klin Farmakol 57:8–11.

Kramer ML, Schulz-Schaeffer WJ. 2007. Presynaptic α-synuclein aggregates, not Lewy bodies, cause neurodegeneration in dementia with Lewy bodies. J Neurosci 27:1405–1410.

Li W, Antuono PG, Xie C, Chen G, Jones JL, Ward BD, Franczak MB, Goveas JS, Li SJ. 2012. Changes in regional cerebral blood flow and functional connectivity in the cholinergic pathway associated with cognitive performance in subjects with mild Alzheimer's disease after 12-week donepezil treatment. Neuroimage 60:1083–1091.

Li Z, Ren A, Zhang L, Guo Z. 2006. A population-based case–control study of risk factors for neural tube defects in four high-prevalence areas of Shanxi province, China. Paediatr Perinat Epidemiol 20:43–53.

Liu CL, Chen H, Jiang Y, Tu PF, Zhong M, Ma JY, Ding H, Zhang WX, Jin XM. 2013. Effects of echinacoside on extracellular acetylcholine and choline levels of hippocampus and striatum of cerebral ischemia rats. Yao Xue Xue Bao 48:790–793.

Masliah E, Ellisman M, Carragher B, Mallory M, Young S, Hansen L, DeTeresa R, Terry RD. 1992. Three-dimensional analysis of the relationship between synaptic pathology and neuropil threads in Alzheimer disease. J Neuropathol Exp Neurol 51:404–414.

Mirvish SS. 1985. Gastric cancer and salivary nitrate and nitrite. Nature 315:461.

Rowell JJ, Mallik AK, Dugas-Ford J, Ragsdale CW. 2010. Molecular analysis of neocortical layer structure in the ferret. J Comp Neurol 518:3272–3289.

Schmidt CA, Clark MW. 2012. Evaluation of a denitrification wall to reduce surface water nitrogen loads. J Environ Qual 41: 724–731.

Scott DA, Tabarean I, Tang Y, Cartier A, Masliah E, Roy SA. 2010. pathologic cascade leading to synaptic dysfunction in α-synuclein-induced neurodegeneration. J Neurosci 30:8083–8095.

Scragg RK, Dorsch MM, McMichael AJ, Baghurst PA. 1982. Birth defects and household water supply. Epidemiological studies in the Mount Gambier region of South Australia. Med J Aust 2:577–579.

Sherwood CC, Duka T, Stimpson CD, Schenker NM, Garrison AR, Schapiro SJ, Baze WB, McArthur MJ, Erwin JM, Hof PR, Hopkins WD. 2010. Neocortical synaptophysin asymmetry and behavioral lateralization in chimpanzees (*Pan troglodytes*). Eur J Neurosci 31:1456–1464.

Shiva S, Sack MN, Greer JJ, Duranski M, Ringwood LA, Burwell L, Wang X, MacArthur PH, Shoja A, Raghavachari N, Calvert JW, Brookes PS, Lefer DJ, Gladwin MT. 2007. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. J Exp Med 204:2089–2102.

Su JJ, Xie, HJ, Zhao WW, Chen YF, Cui ZJ, Wang L, Liu HL, Fan WJ, Deng JB, Deng JX. 2003. Study on C-synuclein gene in patients with idiopathic Parkinson's disease. Chin J M Ed Genet 20:444–446. (Chinese, English abstract).

Surguchov A. 2013. Synucleins: Are they two-edged swords. J Neurosci Res 1:161–166.

Tinsley CJ, Narduzzo KE, Ho JW, Barker GR, Brown MW, Warburton EC. 2009. A role for calcium-calmodulin-dependent protein kinase II in the consolidation of visual object recognition memory. Eur J Neurosci 30:1128–1139.

Totzeck M, Schicho A, Stock P, Kelm M, Rassaf T, Hendgen-Cotta UB. 2015. Nitrite circumvents canonical cGMP signaling to enhance proliferation of myocyte precursor cells. Mol Cell Biochem 401(1–2):175–183.

Toya Y, Nakahigashi K, Tomita M, Shimizu K. 2012. Metabolic regulation analysis of wild-type and arcA mutant *Escherichia coli* under nitrate conditions using different levels of omics data. Mol Biosyst 8:2593–2604.

Vargas KJ, Makani S, Davis T, Westphal CH, Castillo PE, Chandra SS. 2014. Synucleins regulate the kinetics of synaptic vesicle endocytosis. J Neurosci 34:9364–9376.

Vorhees CV, Williams MT. 2014. Value of water mazes for assessing spatial and egocentric learning and memory in rodent basic research and regulatory studies. Neurotoxicol Teratol 45: 75–90.

Wang YY, Hsu PK, Tsay YF. 2012. Uptake, allocation and signaling of nitrate. Trends Plant Sci 17:458–467.

Winchester PD, Huskins J, Ying J. 2009. Agrichemicals in surface water and birth defects in the United States. Acta Paediatr 98: 664–669.

Yoo JW, Lyu J, Lee SD, Kim WS, Kim DS, Shim TS. 2013. Clinical experience of using clofazimine to treat multidrug-resistant tuberculosis. Int J Tuberc Lung Dis 17:1243–1244.

Zhang H, Maitta RW, Bhattacharyya PK, Florea AD, Sen F, Wang Q, Ratech H. 2011. γSynuclein is a promising new marker for staining reactive follicular dendritic cells, follicular dendritic cell sarcoma, Kaposi sarcoma, and benign and malignant vascular tumors. Am J Surg Pathol 35:1857–1865.

Zhang Y, Ye C, Wang A, Zhu X, Chen C, Xian J, Sun Z. 2015. Isolated and combined exposure to ammonia and nitrite in giant freshwater pawn (*Macrobrachium rosenbergii*): Effects on the oxidative stress, antioxidant enzymatic activities and apoptosis in haemocytes. Ecotoxicology 24:1601–1610.

Zhong SC, Luo X, Chen XS, Cai QY, Liu J, Chen XH, Yao ZX. 2010. Expression and subcellular location of alpha-synuclein during mouse-embryonic development. Cell Mol Neurobiol 30: 469–482.