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

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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. 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...
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 immuno-fluorescent 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-FOX2 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 antimyelinated 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 Olympus laser confocal microscope (FV1000; Olympus, Japan) with separate scans 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 using standard curves. The proteins were electrophoresed 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/μm²); ⑤ Density of γ-synuclein positive neuron = the number of γ-synuclein positive cells/cortical area (cells/mm²); 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 (± 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 ATITTQ assays) was compared among various groups. Both FAP and ATITTQ 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 neuron. 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 immunocytochemistry [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
Fig. 2.

Environmental Toxicology DOI 10.1002/tox
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 synaptic 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 multifaceted. 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 (Scrugg 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 (Winchecuter 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...
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. 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.

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.
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 ultrastructure were analyzed with DiI diolistic assay and transmission electron microscopy. We found that nitrite exposure also made dendritic 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$^{2+}$ 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$^{2+}$ 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 (Vargas 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 pre-synaptic 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 \( \gamma \)-synuclein in visual cortex with immunolabeling and tested the correlation among them as well. We found that after nitrite exposure, \( \gamma \)-synuclein positive cells were increased in a dose-dependent manner, suggesting an abnormal accumulation of overexpression of \( \gamma \)-synuclein after nitrite exposure. The overexpression of \( \gamma \)-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 \( \gamma \)-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.

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