DISTURBANCES OF SOLUBLE N-ETHYLMALEIMIDE-SENSITIVE FACTOR ATTACHMENT PROTEINS IN HIPPOCAMPAL SYNAPTOSOMES CONTRIBUTE TO COGNITIVE IMPAIRMENT AFTER REPETITIVE FORMALDEHYDE INHALATION IN MALE RATS

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Abstract—SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein) complex, a four-helical bundle composed of syntaxin1 and synaptosome-associated protein 25 (SNAP25) on the plasma membrane and synaptobrevin/VAMP2 (vesicle-associated membrane protein 2) on the vesicle membrane, plays a key role in synaptic exocytosis and facilitates neurotransmission. Disturbances of SNARE proteins were uncovered in some neurodegenerative diseases, neuroendocrine disturbances and even after environmental interventions. In the present study, we evaluated the effects of formaldehyde (FA) inhalation (13.5 ± 1.5 ppm, twice 30-min each day for two rounds of 14 consecutive days) on learning and memory in Morris water maze and thereafter explored the SNARE protein levels in hippocampal synaptosomes. The formaldehyde-treated rats showed learning and memory impairment in escape latency and probe trials, without mobility disturbances in Morris water maze. Using western blotting assays, we detected the SNARE proteins in hippocampal synaptosomes and identified decrease of both SNAP25 and VAMP2 after formaldehyde treatment without significant changes of another SNARE protein, syntaxin 1, and synaptic vesicle marker, synaptophysin. Furthermore, the neuronal morphology and number detected in Nissl stain and western blotting assay of neurofilament-150 and synaptophysin were not affected after FA treatment. These results suggested that the specific decrease of SNAP25 and VAMP2 in hippocampal synaptosomes served as a potential contributing mechanism underlying learning and memory impairments after repetitive formaldehyde inhalation treatment. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: hippocampal synaptosomes, SNARE proteins, cognitive impairment, formaldehyde inhalation.

Neuronal synaptic vesicle fusion is driven by assembly of the soluble N-ethylmaleimide-sensitive factor attachment protein (SNARE) complex, a four-helical bundle composed of syntaxin1 and synaptosome-associated protein 25 (SNAP25) on the plasma membrane and synaptobrevin/VAMP2 on the vesicle membrane (Verhage and Toonen, 2007). Targeted deletion of SNAP25 and VAMP2 in mice resulted in not only neurotransmission impairment, but also embryonic or prenatal death (Schoch et al., 2001; Washbourne et al., 2002). Previous studies have revealed preferential disturbances of SNARE proteins in specific brain regions in patients with neurodegenerative diseases, such as Alzheimer’s disease (AD), Down syndrome (DS), schizophrenia and Creutzfeldt–Jakob disease (CJD) (Gabriel et al., 1997; Ferrer et al., 1999; Greber et al., 1999; Halim et al., 2003).

Formaldehyde (FA) is widely used in industry in lubricants, adhesives, fertilizers, germicides, dyes, and disinfectants. Formaldehyde is also toxic to mammals, leading to mutagenicity, genotoxicity, sensory irritation or neurotoxicity, carcinogenicity, and even learning and memory impairment (Naya and Nakanishi, 2005; Arts et al., 2006). Many studies have been undertaken on the impacts of formaldehyde on the CNS (Sorg, 1999). Epidemiological data showed the histology technicians exhibited reduced performance on story memory, digit span, pegboard and sharpened-Romberg, increased errors on trials (Kilburn et al., 1985, 1987) and they presented excessive fatigue and difficulty in remembering (Kilburn et al., 1985; Kilburn and Warshaw, 1992; Kilburn, 1994). Animal studies showed interventional effects of FA exposure on psychology and cognition in the water labyrinth (Makowski and Ordonez, 1981; Malek et al., 2003b). Although the impact of FA inhalation exposure on learning and memory was established in labyrinth test, the results in Morris water maze were still unidentified. And the underlying mechanisms were still unclear. A recent proteomic study reported a decrease in levels of SNAP23, a homologue of SNAP25, in rat plasma after formaldehyde inhalation (Im et al., 2006). It suggested that the SNARE protein disturbances might underlie the effects of formaldehyde exposure.

In this paper, we explored the effects of FA inhalation by rats for 3, 7, 14 and 28 days on learning and memory. Compared to control rats, the 3, 7 and 14-days formaldehyde treated rats turned out mild changes in Morris water maze. 28-days formaldehyde-treated rats showed significant learning and memory impairment in escape latency and probe trials without mobility disturbances in Morris water maze. Western blotting assay of hippocampal syn-
aptosomes of 28-days treated rats revealed decrease of SNAP25 and VAMP2 without significant changes of another SNARE protein, syntaxin 1, and a synaptic vesicle marker, synaptophysin. Furthermore, the Nissl stain and western blotting assay of neurofilament-150 and synaptophysin in hippocampus suggested normal neuronal morphology and number after FA treatment for 28 days in our paradigm. These results substantiated the hypothesis that SNARE protein disturbances in hippocampal synaptosomes could contribute to learning and memory impairment after repetitive FA inhalation.

EXPERIMENTAL PROCEDURES

Animals
Male Sprague–Dawley (SD) rats ranging from 160 to 180 g were obtained from the Institute of Animal care of Health Science Center of Peking University (Beijing, PR China). They were randomly group-housed in a standard animal care room with the temperature 22±1 °C, the humidity 50±5%, and 12-h light/dark cycle. The rats acclimatized to the environment and were provided with chow and water ad libitum except when they were placed into the exposure chambers or the behavioral testing apparatus. The rats were divided into groups by their performances in probe trials after grouping training session in Morris water maze as described below.

Formaldehyde inhalation exposure
Formaldehyde gas was generated by evaporation from a formalin solution (37%, Beijing Chemical Works, Beijing, China) which was added onto the four lower insides of the static toxification chambers (54 cm×31 cm×34 cm), with a concentration of around 13.5±1.5 ppm as determined by using a Formaldehyde Detect Device (Interscan 4160-2, Chatsworth, CA, USA). The rats were exposed to formaldehyde twice a day for 30 min each time (7:00–7:30 AM and 19:00–19:30 PM). When the formaldehyde treated group was exposed for 30 min, the controls were exposed for 30 min with fresh air in the chamber. Between the treatments, the chambers were cleaned up. We recorded the food and water intakes as well as the body weights of rats.

All experiments were conducted following an approved protocol from the animal care committee of the Peking University and performed in accordance with the animal care guidelines of the Chinese Council.

Morris water maze test

Apparatus. The Morris water maze test was conducted according to Morris (Morris 1984). The water-filled (23±1 °C) black-colored tank (150 cm diameter; 60 cm depth) was divided into four quadrants of equal area arbitrarily. A circular platform (10 cm in diameter) made of transparent Perspex was submerged 1 cm below the water surface with its center 37.5 cm from the perimeter, in the middle of one quadrant (the target quadrant). A closed-circuit television camera was mounted onto the ceiling directly above the center of the pool to convey subject swimming trajectories and parameters to an electronic image analyser.

Procedure. Habituation. The Morris water maze test began with one day habituation. The rats were forced to stay on the platform for 45 s, which was 1 cm beneath the water. If the rats jumped down, they would be put back onto the platform, meanwhile the time record re-started.

Grouping session (training session 1–3). In order to minimize innate differences between animals, which would mix up the performances in water maze, we grouped the rats (eight in control and 11 in formaldehyde treatment group) by their performances in the probe trial following three sessions of hidden platform training. Each session contained three trials, and the interval between two trials was 30 s. The rats in two groups were confirmed to perform comparably in the probe trial before the treated group accepted formaldehyde inhalation exposure.

Hidden platform training and probe trial (training session 4–13). Hidden platform training was performed according to A. L. Markowska et al. (Markowska et al., 1993) with variable-interval probes to evaluate learning and memory repeatedly. On the seventh day of the second round of formaldehyde inhalation treatment, the rats undertook one day habituation as described above. From the following day, the hidden platform training was performed two sessions a day with 4 h between the two for five consecutive days. The rat was placed into the pool facing the perimeter, allowed a maximal time of 90 s to find the platform and step onto it. If the rat crossed the platform without stopping (jumping immediately into the water), it was left to swim on. After finding the platform, the rat was allowed to stay on it for 15 s. If the rat failed to find the platform in the allotted time, it was guided onto the platform and allowed to stay on it for 15 s. After finishing all the three trials, the rat was taken from the platform, gently dried with a towel and returned to its home cage. 4 h later, the other training session started. Between two successive trials, the water was changed in order to erase olfactory traces of previous swim pattern (Means et al., 1992; Maaswinkel and Whishaw, 1999). The platform stayed in the same location throughout the hidden platform training.

The probe trials were performed 1.5 h after training session 5 and 9, and 18 h after training session 7 and 11 to evaluate short-term and long-term memory separately. During the probe trials, the platform was removed from the pool and the rat was given 60 s to swim freely.

Cued platform training (training 14–16). Cued platform trainings were used to exclude the potential contributing effects of eyesight decline caused by formaldehyde exposure on learning and memory impairment in hidden platform training. The procedure was similar to hidden platform training, except that the platform was visible (2 cm above the water surface).

Hidden platform training (session 17). After cued platform training, the rats were tested in hidden platform training again to further testify the effects of formaldehyde inhalation on learning and memory in Morris water maze test.

Isolation of hippocampal synaptosomes and western blotting analysis

One day after the Morris water maze test, the rats were decapitated after anesthesia by i.p. injection of chloral hydrate (350 mg/kg). Synaptosome isolation was performed according to Dunkley et al. (Dunkley et al., 2008). In short, the hippocampus lysates were centrifuged at 1000 g at 4 °C for 10 min after isosmotic homogenization. The supernatants were centrifuged at 17,000 g at 4 °C for 20 min to obtain the P2 fraction for further percoll (Pharmacia Biotech, Uppsala, Sweden) gradient separation. In order to obtain more synaptosomes, we collected the fractions between 10% and 23% for western blotting analysis. The final pellets were centrifuged at 17,000 g at 4 °C for 20 min twice and resuspended with Tris–HCl buffer (pH 7.4) to remove residual Percoll.

The synaptosomal protein concentration was determined by BCA assay (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. Both protein samples of hippocampus synaptosomes (8 μg) prepared as described above and the RIPA lysed hippocampus total protein (20 μg) were boiled in loading buffer for 5 min and subjected to 12% SDS–PAGE.

The separated proteins were then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membrane was blocked in 5% skim milk in TBST (0.075%) for 1 h at room
temperature and then incubated with primary antibodies in TBST (0.075%) at 4 °C overnight. The antibodies dilutions were 1:4000 for SNAP25 (BD Biosciences, San Jose, CA, USA), 1:4000 for VAMP2 (Abcam, Cambridge, UK), 1:4000 for syntaxin1 (Santa Cruz Biotechnology, CA, USA), 1:2000 for synaptophysin (MBL, Nagoya, Japan), 1:1000 for TuJ-1 (Abcam, Cambridge, UK) and 1:1000 for neurofilament (150 kD, Chemicon, Temecula, CA, USA). After washing three times in TBST for 10 min, the membrane was washed and detected through chemiluminescence (ECL, pierce, Rockford, IL, USA).

Nissl stain

Tissue preparation and Nissl stain were performed as described (Chui et al., 1992).

Data analysis

All data were expressed as group means±standard error of the mean (SEM). Data from Morris water maze test (escape latency) were subjected to a non-parametric ANOVA followed by Turkey’s multiple comparison test: compare all pairs of columns for multiple pairs of data. Data from food and water intakes, body weight, Morris water maze test (probe trial) and western blotting analysis were evaluated by unpaired T-test. Statistical difference was set at P<0.05.

RESULTS

Physical measurements of rats throughout formaldehyde treatment

During FA treatment, we monitored the food and water intakes (data not shown) and weights of body and some endocrine tissues (shown in Table 1). There were no significant differences between the control and FA treated rats.

Learning and memory impairment in Morris water maze

Firstly, we explored the effects of short-term exposure to FA (3, 7 and 14-day) on learning and memory in Morris water maze with data not shown. Based on comparative performances in grouping session, the escape latency in hidden platform training session revealed similar results between control and FA treated rats. After the training session in Morris water maze, the control and 3-day formaldehyde treated rats spent significantly increased time and distance in platform quadrant in the probe trials compared to those data in probe trials of grouping session, meanwhile the 7 and 14-day groups showed similar results. What’s more, the control rats crossed significantly more times in center region compared to that in probe trials of grouping session, whereas all the FA treated rats performed similar even after training sessions. The results above suggested that the inhalative formaldehyde treatment (3, 7, 14-day) could potentially affect learning and memory in Morris water maze.

Furthermore, we treated the rats with FA exposure for 28 days and made a more detailed Morris water maze test. Based on the comparative performances in the grouping sessions (Fig. 1A, session 1–3), the formaldehyde treated rats spent more time than the control rats to find the platform with increasing latency (P<0.05) as the formaldehyde treatment proceeded (Fig. 1A, session 10–12). In the following cued platform training (Fig. 1A, session 14–16), which aimed to exclude the potential contributing effects of rat’s eyesight impairment caused by formaldehyde treatment on learning and memory impairment in Morris water maze, formaldehyde treated rats showed highly similar performances compared to control rats. Then we evaluated the hidden platform training again for one session to identify the effects of formaldehyde on learning and memory. The results, though not significant, clearly demonstrated a tendency for learning and memory impairment after inhalation treatment of formaldehyde (Fig. 1A, session 17).

During hidden platform training, we performed repeated probe trials, 1.5 h after training session 5 and 9 as indicated by the arrows, and 18 h after training session 7 and 11 as indicated by the arrowheads (Fig. 1A), to evaluate short-term and long-term memory respectively. Compared to the similar performances in probe trials before FA treatment (Fig. 1B, C), the time and distance spent in platform’s quadrant (PQ), platform quadrant/all quadrants, after training session 5 in short-term probe trial (STP) presented short-term learning and memory impairment with P<0.05 (Fig. 1B, C, STP). Similar results could be found 18 h after training session 11 in long-term probe trial (LTP) which showed long-term learning and memory impairment after FA treatment with P<0.01 (Fig. 1B, C, LTP). Meanwhile, the formaldehyde treatment seemed to have no significant effects on mobile activity reflected by similar swimming speed (Fig 1D) and swimming distance (Fig 1E) of the rats before and after FA treatment in the probe trials.

Specific SNARE protein disturbances in hippocampal synaptosomes with unaffected neuronal morphology and number

We detected SNARE proteins in hippocampal synaptosomes using western blotting analysis after the formala-
In the present study, we evaluated the effects of FA inhalation (13.5±1.5 ppm, twice 30 min each day) on learning and memory in Morris water maze and thereafter explored the SNARE protein levels in hippocampal synaptosomes. The FA-treated rats showed learning and memory impairment in escape latency and probe trials without mobility disturbances in Morris water maze test. We also identified significant decrease of SNAP25 and VAMP2 proteins after formaldehyde treatment in our paradigm without significant changes of another SNARE protein, syntaxin 1, and synaptic vesicle marker, synaptophysin. Furthermore, the neuronal number revealed by western blotting of neurofilament-150 and synaptophysin, and neuronal morphology in Nissl stain of hippocampus was not affected after formaldehyde treatment.

Malek and his colleagues have identified learning and memory impairment in water labyrinth test, as well as psychological disturbances in open field test utilizing similar FA inhalation treatment to ours (Malek et al., 2003a, b). Several other studies also found consistent results on CNS disturbances after formaldehyde exposure, such as mood disturbances.
changes, mnemonic difficulties, fear conditioning in human and animal models (Kilburn et al., 1987; Hawkins et al., 1994; Sorg et al., 2004). However, controversy still existed in different behavioral tests including Morris water maze test and elevated plus maze test. It could be due to the various exposure types, doses of FA, and strains of rodents used. In certain kinds of job, people would have to be exposed to high doses of formaldehyde both occasionally and repeatedly. Hence, we evaluated the effects of formaldehyde inhalation exposure using the following paradigm: (1) FA inhalation, around 13.5±1.5 ppm, which was a high dose (previous studies have used formaldehyde below 11 ppm to find the psychological and psychiatric changes and memory impairment); (2) treatment twice each day, 30 min each time. Considering the high-dose, long treatment duration each time could trigger habituation, we confined the treatment duration to a short time (3) we detected the effects of different formaldehyde inhalation exposure to rats by well designed Morris water maze to evaluate various learning and memory impairment.

Many studies have investigated mechanisms underlying the behavioral changes, such as neurotransmitter and endocrine system changes. Repeated formaldehyde exposure in rodents produced CNS plasticity manifest as greater sensitivity to dopaminergic drugs (cocaine) (Sorg et al., 1998; Sorg and Hochstatter, 1999). Inhalation exposure of formaldehyde selectively regulated mRNA of N-methyl-D-aspartate (NMDA) receptor subunits (NR2A) and dopamine receptor subtypes D1 and D2 (Ahmed et al., 2007). Hypothalamus-pituitary axis played essential roles in psychology and psychiatry and even memory impairment (Sauro et al., 2003; Swaab et al., 2005). Exposure to formaldehyde, acute or repeated, would lead to disturbances of many HPA related hormones such as triiodothyronine (T3), thyroxine (T4) thyroid stimulating hormone (TSH), ACTH, and corticosterone (Sorg et al., 2001; Patel et al., 2003; Sari et al., 2004, 2005). The perinatal hypothyroidism induced by propylthiouracil (PTU) through gavage to pregnant rats reduced mRNA and protein level of SNAP25 in neonatal brain (Zhang et al., 2008). Furthermore, a recent study used proteomic analysis to revealed reduced SNAP23, a homologue of SNAP25, in rat plasma after long-term formaldehyde inhalation (Im et al., 2006).

Decrease of SNAP25 usually served as a marker for neuronal loss or impaired synaptogenesis. Osten Sand has employed an antisense oligonucleotide which was complementary to coding regions to reduce SNAP25 expression in cortical neurons in culture, resulting in decreased axonal outgrowth and nerve growth factor (NGF)-induced neurite elongation (Osen-Sand et al., 1993). VAMP2 protein expression decreased in prefrontal cortex of schizophrenia patients compared with controls, which suggested the discrete changes of VAMP2 among the variable vesicle proteins (Halim et al., 2003). In this paper, we identified protein decreases of SNAP25 and VAMP2 without the changes of syntaxin1, synaptic marker synaptophysin and neuronal marker Tuj-1 in hippocampus synaptosomes. These results suggested that SNAP25 and

**Fig. 2.** SNAP25 and VAMP2 protein reduced in hippocampal synaptosomes after formaldehyde inhalation exposure. Western blotting and quantitative analysis of Tuj-1(a), synaptophysin(b), Syntaxin 1(c), SNAP25(d) and VAMP2(e) are presented in (A, B) respectively. Ct, control; FA, formaldehyde. Control, n=5. Formaldehyde treated group, n=8. * P<0.05, ** P<0.01. Error bar means SEM.
VAMP2 changes in nerve terminals were early events before neuronal loss. The changes were most likely to cause changes in neurotransmission.

Endogenous formaldehyde, produced from diet and environmental exposure, as well as some prodrugs, was dynamically metabolized in human body (Dhareshwar and Stella, 2008). Clinical investigations revealed that blood formaldehyde concentration increased with aging (above 60). Furthermore, the morning uric formaldehyde was found to be positively related to the degree of dementia: the more severe the dementia, the higher the concentration of uric formaldehyde of the patients (He et al., 2009). These all suggested the contributing effects of formaldehyde exposure on learning and memory impairment.

In the current study, we evaluated the effects of formaldehyde inhalation treatment on learning and memory in Morris water maze. The following western blotting revealed disturbances of SNARE proteins in hippocampal synaptosomes without synaptic vesicle loss and neuron changes. The results implicated the specific decrease of SNAP25 and VAMP2 proteins in hippocampal synaptosomes as a potential contributing mechanism underlying learning and memory impairment after repetitive formaldehyde inhalation treatment.

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