Research Report

Impulse noise exposure in rats causes cognitive deficits and changes in hippocampal neurotransmitter signaling and tau phosphorylation

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ABSTRACT

Noise exposure has been characterized as a stressor, and its non-auditory effects on the central nervous system have been established both epidemiologically and experimentally. Little is known about the impact of impulse noise on the brain, however. In this study, we examined the effects of impulse noise stress on spatial learning and memory and on associated changes in the hippocampus. Rats were exposed to 20 sound impulses with a peak sound pressure of 165 dB and duration of 100 ms. Impulse noise stress led to a temporary decrease in cognitive function as evidenced by poor spatial memory in the Morris water maze (MWM). Effects of noise on the glutamate (Glu)-N-methyl-D-aspartic acid receptor (NMDAR) signaling system and hippocampal tau phosphorylation were investigated by high performance liquid chromatography, Western blotting, and immunohistochemistry. The concentrations of Glu and aspartate (Asp) in the hippocampus were increased at 30 min after exposure and remained elevated for the entire observation period (24 h), while the content of glycine (Gly) was stable for several hours following noise but also increased by 24 h after noise stress. Impulse noise stress also caused a significant increase in NMDAR 2B subunit (NR2B) expression and a two-phase increase in tau phosphorylation in hippocampus. Immunohistochemistry confirmed tau hyperphosphorylation in hippocampus that was most prominent in the dentate gyrus (DG) and CA1 region. These findings demonstrate that impulse noise stress impairs early spatial memory, possibly by disrupting Glu-NMDAR signaling and triggering aberrant tau hyperphosphorylation in hippocampus.

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1. Introduction

Impulse noise is defined as noise consisting of single bursts with a duration of less than one second and peak levels 15 dB higher than background noise. It is exemplified by such environment sounds as hammering, stamping, or gunfire. Impulse noise may be more harmful than continuous, steady-state noise. Indeed, impulse noise exposure may lead to neurological damage beyond auditory pathways to affect other areas of the CNS (Cernak et al., 1999). High level impulse noise of 198 dB or 202 dB has been shown to alter c-Jun, c-Fos, and c-Myc expression, induce deposition of amyloidogenic β-APP, increase TUNEL reactivity (a sign of neuronal apoptosis), and cause the redistribution of neurofilament subunits in cor-

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tical and hippocampal neurons (Säljö et al., 2002a, 2002b). It is thought that impulse noise with a peak sound pressure level higher than 170.4 dB may generate dynamic pressure (GJB 2A-96, 1996) and cause blast-induced impairments such as combat-related traumatic brain injury (TBI) (Hoge et al., 2008; Leung et al., 2008). Exposure to higher levels (Pmax 100–300 kPa) causes significant injury to both neuronal and glial cells, as well as brain edema (Cernak et al., 1997; Kaur et al., 1995; Säljö et al., 2000, 2002a, 2002b). However, the level of impulse noise under 170 dB generally experienced by humans (Flamme et al., 2009a, 2009b) cannot cause blast, and the effects of non-blast impulse noise exposure on cognitive function are largely unknown. The goal of the present study is to investigate whether impulse noise lower than 170.4 dB negatively impacted cognitive function and caused alterations in the hippocampus, a key brain region related to some forms of cognition.

Among cognitive functions, reading, attention, problem solving, and memory are most strongly affected by noise (WHO, 2000). Spatial cognition is coordinated by different brain regions, but is highly dependent on the hippocampus. A recent animal study demonstrated the scope of different effects induced by chronic noise exposure, including oxidative stress, increased acetylcholinesterase activity, reduced NMDA receptor subunit NR2B in rat hippocampus induced by chronic noise exposure might have caused these impairments in spatial learning and memory (Cui et al., 2009). Experience has shown that impulse noise may be more harmful to hearing than continuous, steady state noise (Mantysalo and Vuori, 1984). However, little is known about the differences in the effects between both forms of noise exposure on cognition. The amino acid neurotransmitter system, especially glutamate and its receptors, has been implicated in cognition and in cognitive disorders. Glutamate is the principal transmitter mediating fast excitatory synaptic responses in the vertebrate CNS (Collingridge and Lester, 1989), and NMDARs constitute a major class of Glu receptors in the mammalian brain (Dingledine et al., 1999; Hollmann and Heinemann, 1994). The NMDA receptor is a heteromeric ligand-gated ion channel that mediates synaptoplastic functions such as long-term potentiation (LTP) and long-term depression (LTD) (Cull-Candy et al., 2001; Lijam et al., 1997). The NR2B subunit is a critical structural and functional component of the NMDA receptor and plays a major role in learning and memory (Moon et al., 1994; Tang et al., 1999). The protein tau is central to the dynamics of microtubule assembly, and hence the maintenance of neuronal morphology. Hyperphosphorylation of tau impairs cytoskeletal function by disrupting microtubule assembly (Alonso et al., 1997). In addition, hyperphosphorylated tau inclusions are a common pathological hallmark of several neurodegenerative disorders (Iqbal et al., 2005). Impulse noise redistributes phosphorylated neurofilament subunits in neurons and elevates β-APP, another marker of neurodegeneration or neuronal damage (Säljö et al., 2000, 2002b).

To investigate the effects of impulse noise exposure on cognition, hippocampus-dependent spatial memory was assessed in the Morris water maze (MWM). To determine whether impulse noise induced neuropahtological changes in the hippocampus, high performance liquid chromatography (HPLC) was used to measure the concentration of amino acid neurotransmitters. Immunoblotting and immunohistochemistry were employed to reveal the expression and distribution of NR2B and phospho-tau in the hippocampus.

2. Results

2.1. MWM test

The three-day training period (12 trials) led to a significant decrease in the average latency to find the hidden platform in the MWM, from approximately 45 s on day 1 to approximately 15 s on day 3 (data not shown). After impulse noise exposure on day 4, escape latencies in the hidden platform test exhibited an upward trend that did not reach statistical significance.
(data not shown). However, the time spent in the target quadrant during the probe trial was significantly lower in rats 30 min and 3 h following impulse noise exposure compared with MWM-trained controls (noise exposed: 26.6 s at 30 min and 30.1 s at 3 h; control: 37.7 s; \(p < 0.05\) ) (Fig. 1A). Similarly, the number of times the rats crossed the former platform location was significantly reduced by noise exposure (noise exposed: 2.3 time at 30 min and 3.0 times at 3 h; control: 5.1; \(p < 0.05\) ) (Fig. 1B). No significant difference in swimming speed occurred between control and all impulse noise exposure groups (Table 1).

2.2. Amino acid neurotransmitters

As shown in Table 2, the concentrations of Glu and Asp in the hippocampus increased by about two fold 30 min after impulse noise exposure, continued to increase over the next 3–6 h, and remained elevated 24 h after exposure. Impulse noise exposure also caused a significant increase in the level of Gly 24 h following exposure. In contrast, the levels of GABA were not significantly different at any observed time point.

2.3. Immunoblot analysis of NR2B and p-Tau

The expression of the NR2B protein and changes in the phosphorylation tau were examined by quantitative immunoblot analysis of hippocampal extracts from individual rats sacrificed at 30 min, 3 h, 6 h, and 24 h after impulse noise exposure. The expression of NR2B in hippocampus was significantly elevated at 30 min after noise exposure relative to unexposed controls, and this elevated expression was maintained during the entire observation period (Fig. 2). The hippocampal expression of p-Tau (Thr205) in hippocampus increased at 30 min after impulse noise exposure but decreased at 3 h. Expression of p-Tau was elevated again at 6 h and 24 h, indicating that impulse noise exposure caused dynamic changes of phosphorylated tau at Thr205 in hippocampus (Fig. 3).

2.4. p-Tau immunoreactivity

To determine the distribution patterns of p-Tau (Thr205) in the different regions of hippocampus, we analyzed sections of hippocampus by immunohistochemistry. Phospho-tau immunoreactivity was observed in the DG, CA1, and CA3, but at fairly low levels in the hippocampi from control rats (Fig. 4 A1, A2, A3, and F). In noise-exposed rats, however, p-Tau (Thr205) immunoreactivity was substantially higher in CA1 and CA3 pyramidal cell layers, and in the DG granule cell layer compared with control rats. Distinct increases in p-Tau (Thr205) immunoreactivity were detected at 30 min after impulse noise exposure (Fig. 4 B1, B2, B3, and F). p-Tau (Thr205) immunoreactivity increased only in CA3 (Fig. 4 C1, C2, C3, and F) at 3 h after exposure, but markedly elevated again in DG, CA1 and CA3 at 6 h and 24 h after noise exposure compared with sections from control rats (Fig. 4 D1-3, E1-3, and F). Thus, our immunohistochemical staining results mirrored the results from immunoblotting.

3. Discussion

The present study demonstrated that impulse noise exposure interfered with spatial learning and memory, and altered amino acid neurotransmitter levels, NR2B expression, and p-Tau immunoreactivity was observed in the DG, CA1, and CA3, but at fairly low levels in the hippocampi from control rats (Fig. 4 A1, A2, A3, and F). In noise-exposed rats, however, p-Tau (Thr205) immunoreactivity was substantially higher in CA1 and CA3 pyramidal cell layers, and in the DG granule cell layer compared with control rats. Distinct increases in p-Tau (Thr205) immunoreactivity were detected at 30 min after impulse noise exposure (Fig. 4 B1, B2, B3, and F). p-Tau (Thr205) immunoreactivity increased only in CA3 (Fig. 4 C1, C2, C3, and F) at 3 h after exposure, but markedly elevated again in DG, CA1 and CA3 at 6 h and 24 h after noise exposure compared with sections from control rats (Fig. 4 D1-3, E1-3, and F). Thus, our immunohistochemical staining results mirrored the results from immunoblotting.

**Table 1** - Latency to platform, path length and swimming speed of rats in MWM test after impulse noise exposure (INE) (means ± S.D., \(n = 8\) for each condition).

<table>
<thead>
<tr>
<th>Group</th>
<th>Latency (s)</th>
<th>Path length (cm)</th>
<th>Swimming speed (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.6±2.2</td>
<td>377.1±50.7</td>
<td>24.7±3.8</td>
</tr>
<tr>
<td>30 min after INE</td>
<td>19.0±3.1</td>
<td>569.8±79.2</td>
<td>25.0±3.9</td>
</tr>
<tr>
<td>INE</td>
<td>15.0±2.9</td>
<td>427.4±55.6</td>
<td>23.9±4.1</td>
</tr>
<tr>
<td>6 h after INE</td>
<td>14.1±2.6</td>
<td>397.1±55.0</td>
<td>24.8±3.7</td>
</tr>
<tr>
<td>24 h after INE</td>
<td>13.7±2.3</td>
<td>405.6±53.8</td>
<td>25.1±4.0</td>
</tr>
</tbody>
</table>

**Table 2** - The effect of impulse noise exposure (INE) on the total level of several amino acid neurotransmitters in the hippocampus (means ± S.D., \(n = 8\) for each condition).

<table>
<thead>
<tr>
<th>Group</th>
<th>Glu (μmol/g)</th>
<th>Asp (μmol/g)</th>
<th>Gly (μmol/g)</th>
<th>GABA (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.18±1.32</td>
<td>2.71±0.25</td>
<td>1.34±0.40</td>
<td>3.64±0.95</td>
</tr>
<tr>
<td>30 min after INE</td>
<td>29.34±2.01**</td>
<td>8.96±1.04**</td>
<td>1.49±0.15</td>
<td>3.07±0.34</td>
</tr>
<tr>
<td>3 h after INE</td>
<td>29.12±1.99**</td>
<td>9.09±1.11**</td>
<td>1.66±0.17</td>
<td>3.30±0.39</td>
</tr>
<tr>
<td>6 h after INE</td>
<td>28.96±1.47**</td>
<td>9.03±1.07**</td>
<td>1.53±0.16</td>
<td>3.47±0.26</td>
</tr>
<tr>
<td>24 h after INE</td>
<td>29.79±3.60**</td>
<td>8.56±1.12**</td>
<td>1.96±0.22**</td>
<td>3.49±0.61</td>
</tr>
</tbody>
</table>

*\(p < 0.05\) and **\(p < 0.01\) vs. controls.

**Fig. 2** - Immunoblot showing increased NR2B expression in the hippocampus after impulse noise exposure. GAPDH was used as the internal reference protein. The density of immunoreactive bands was quantified and presented as percent change relative to control samples. Bars represent mean ± S.D. (\(n = 6\) for each condition). *\(p < 0.05\) and **\(p < 0.01\) vs. controls.
Tau (Thr205) in the CA1, CA3, and DG regions of hippocampus. The MWM is a widely used test of spatial learning and memory. Performance in the MWM involves several components, including concept formation (learning the general rules of the task), attention, working memory, and reference memory. The hidden-platform test and probe trial test are considered to be the most specific tests for spatial learning and memory, respectively (Morris et al., 1982). Our findings revealed that impulse noise exposure impaired early memory in the probe trial, but had no significant effect on the hidden platform task (learning), findings in contrast to blast-induced long-term cognitive impairment (Silj6 et al., 2010). Spatial learning and memory are highly dependent on hippocampal function, and the hippocampus is prone to stress-induced damage. The hippocampus is involved in the integration of cognitive and emotional information and in modulating hypothalamic-pituitary-adrenal (HPA) responses to psychological stress. Learning and memory show an age-related decline. The hippocampus is involved in the integration of excitatory and inhibitory signals is a basic attribute of neuronal communication, and synaptic plasticity related to learning and memory requires the maintenance of adequate levels of excitation and inhibition. Glutamate is present at concentrations between 5 and 15 μmol/g weight of wet tissue (Erecinska and Silver, 1990; Perry et al., 1987). In this study, the concentration of Glu and Asp in hippocampus increased to 29.34 μmol/g and 8.96 μmol/g respectively at 30 min after impulse noise exposure, and remained elevated for the entire observation period. This increase in excitatory neurotransmitters might have caused an imbalance between excitatory and inhibitory signals and diminished synaptic plasticity, resulting in cognitive impairment. A growing body of evidence suggests that perturbations in systems using the excitatory amino acid Glu may underlie the pathogenic mechanisms of acute brain insults and chronic neurodegenerative disorders. On the other hand, the increase in the inhibitory neurotransmitter GABA at 24 h after exposure may be a compensatory mechanism to reestablish the balance between excitatory and inhibitory signals.

Excitotoxicity is mediated predominantly by NMDA-type Glu receptors (Olney, 1994), which exhibit a higher permeability for Ca²⁺ than do AMPA or KA receptors, and possess a greater capacity for inducing intracellular Ca²⁺ overload and triggering neurodegenerative cascades (Choi, 1992). NMDARs mediate opposing effects depending on their localization. Stimulation of synaptic NMDARs induces prosurvival events, whereas activation of extrasynaptic NMDARs leads to excitotoxic death (Riccio and Ginty, 2002). It has been reported that NR2A-containing NMDARs are predominantly confined to synapses, whereas NR2B-containing NMDARs are preferentially distributed extrasynaptically (Charton et al., 1999). In this study, we found that the expression of NR2B in the hippocampus was significantly increased for at least 24 h after impulse noise exposure. The increase in Glu and Asp, concomitant with over-expression of NR2B-containing NMDARs in the hippocampus, may have led to excitotoxicity in a manner consistent with Alzheimer's disease (AD) neuropathology.

Stressors like food deprivation, cold water swimming, and ether anesthesia cause Alzheimer-like progressive hyperphosphorylation of tau in the brain (Ikeda et al., 2007; Okawa et al., 2003; Yanagisawa et al., 1999). Hyperphosphorylation of tau is usually most prominent in the hippocampus and the initial AD neuropathology is associated with this brain structure (Braak and Braak, 1991; Delacourte et al., 1999). The Thr205 site is one of the major sites on the tau protein that is highly phosphorylated in AD brain (Goedert, 1993). In this study, we showed not only an immediate tau hyperphosphorylation after impulse noise exposure, which replicates other studies (Fujio et al., 2007; Ikeda et al., 2007; Okawa et al., 2003), but also a later phase of tau phosphorylation, suggesting a delayed deleterious effect of impulse noise exposure. We speculate that the late phase of hyperphosphorylation over an 18-h period might be even more harmful to neurons. Studies have shown that dynamic changes in tau phosphorylation after stress are likely the result of a complex regulatory network of protein kinases and phosphatases (Ikeda et al., 2007; Okawa et al., 2003). The early phase of tau hyperphosphorylation may be regulated by rapid reversible changes of immediate response protein kinase, such as JNK (Okawa et al., 2003), while the late phase may relate with slow response mediators of phosphorylation such as GSK-3β and/or protein phosphatase(s). Another intriguing possibility is that a
Fig. 4 – Immunohistochemical staining showing p-Tau (Thr205) expression in the DG (A1, B1, C1, D1, E1), CA1 (A2, B2, C2, D2, E2), and CA3 (A3, B3, C3, D3, E3) regions of the hippocampus in controls (A1-3), and at 30 min (B1-3), 3 h (C1-3), 6 h (D1-3), and 24 h (E1-3) after impulse noise exposure. Bar, 50 μm. Immunoreactivity density was quantified and statistically treated, and is shown in Fig. 4F. Bars represent mean±S.D. (n=6 for each condition). *p<0.05 and **p<0.01 vs. controls.
decrease in phosphorylation at 3 h after noise exposure may be a secondary consequence of alteration of protein phosphatase(s) through some negative feedback regulation. However, to clarify the causal relationships between them, further studies should be performed. Tau hyperphosphorylation in the hippocampus is directly linked to cognitive impairments and neurodegeneration (Spillantini and Goedert, 1998; Sun et al., 2005). Psychological distress is not only a risk factor for AD, but has also been implicated in the memory impairments in AD patients (Wilson et al., 2004, 2005). It seems reasonable to suggest that a stress-related dysregulation of tau phosphorylation in the hippocampus can manifest as memory impairment in the development of diseases such as Alzheimer’s. It has been confirmed that extra-synaptic NR2B-containing NMDARs mediate hyperphosphorylation of tau (Chohan and Iqbal, 2006). The noise-induced abnormality of the EAA-NMDAR signaling system could be involved in hyperphosphorylation of tau, as over-activation of this signaling pathway may cause enhanced kinase activity (Alonso et al., 2004; Chatterjee et al., 2009; Okawaa et al., 2003). Previous studies demonstrated that ether stress and forced cold water swimming caused reversible hyperphosphorylation of tau in the mouse brain (Ikedaa et al., 2007; Okawaa et al., 2003). Notably, more conspicuous hyperphosphorylation was observed at Ser202/Thr205 (AT8 site) and Thr231/Ser235, which are among the prominently phosphorylated sites of PHF-tau in AD brains (Goedert, 1993). Therefore, we suggested that high-intensity impulse noise, as an acute cauchoetic stressor, might also cause hyperphosphorylation of tau at Ser202/Thr205 or Thr231/Ser235, a possibility that will be addressed in a future study.

In summary, the results of the MWM test presented in the current study demonstrate the deleterious effects of impulse noise exposure on early spatial memory. Elevated glu and NR2B and aberrant hyperphosphorylation of tau induced by abnormal EAA-NMDAR signaling in hippocampus correlated with neuronal dysfunction, and these changes may have mediated the observed cognitive deficits. Our work provides evidence for non-auditory effects of impulse noise exposure on the CNS, and the results may be helpful for understanding the underlying neuropathological events and ensuing symptomology.

4. Experimental procedures

4.1. Animals

A total of 100 male Sprague–Dawley rats weighing 200–220 g (6–7 weeks of age) were used in this study. The rats were kept in a room with controlled ambient temperature (23 ± 2 °C), humidity (50%–70%), and a 12-h light/dark cycle (light on from 06:00–18:00 h). They had free access to water and food in their home cages and were allowed to adapt to our laboratory environment for 5 days before the start of experiments. The animal use protocol was approved by the Animal and Human Use in Research Committee of The Institute of Health and Environmental Medicine, Tianjin, PR China. The rats were randomly assigned to either an impulse noise-exposed group or a control group. A total of 80 animals were exposed to impulse noise. At different time point (30 min, 3 h, 6 h, or 24 h) after noise exposure, 8 rats were used in MWM testing and 12 rats were killed by decapitation for other biochemical analyses. The 20 rats (8 for MWM, 12 for sacrifice) that were assigned to the control group were housed in similar cages, but they were not exposed to the noise protocol.

4.2. Impulse noise exposure

The impulse noise was generated by a high level impulse noise generator (Wu et al., 1999) located inside a reverberation chamber. An experimental animal was placed in a wire-mesh cage that was located in the center of the sound field. The animal was positioned on a horizontal shelf with the head towards a high pressure airflow loudspeaker. A B & K 3560 C-size front end and 4938 1/4″ pressure-field microphone were used to record the exposure conditions (Briel and Kjær Instruments, Denmark). Each animal was exposed to twenty, 100 ± 5 ms noise pulses of 165 ± 1 dB (relative to 20 μ Pa) (Fig. 5).

4.3. MWM

A circular pool (diameter, 100 cm; height, 50 cm; depth of water, 32 cm) was used in these experiments. The escape platform (diameter, 10 cm) was 2 cm below the water surface during training. The water was kept at 23 ± 2 °C and made opaque with white comminuted foamed plastics. The pool was situated in a room with visual cues. The animals’ movements were recorded with a video camera attached to the ceiling. The pool was divided into four arbitrary quadrants for probe test analysis. The quadrant in which the platform was located was designated the target quadrant. During task acquisition, a computer program measured the time taken by animals to reach the platform (latency). Animals were placed in the water at one of four starting positions (north, east, south, or west) that alternated in a clockwise manner. Four trials were performed per day, and the inter-trial interval was about 5 min. The cut-off time for a trial, if the animals failed to locate the platform, was 60 s; in such cases the animals were manually placed on the platform for 10 s. The rats were randomly assigned to the noise-exposed group and the control

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group and exposed to impulse noise after 3 days of task acquisition training (12 trials), and then task acquisition (2 trials) and a further probe test (2 trials) were performed 30 min, 3 h, 6 h, and 24 h after impulse noise exposure on day 4. The tests of all groups on day 4 were performed in the following sequence: group of 30 min after noise exposure (15:00–15:30), group of 3 h after noise exposure (15:35–16:00), group of 6 h after noise exposure (16:10–16:35), group of 24 h after noise exposure (16:45–17:10), control group (17:20–17:45). During the probe test, the platform was absent. Each rat was placed in the pool once for 60 s, starting from the same location as the first trial in the hidden platform test. The time spent in the pool once for 60 s, starting from the same location as the former platform location was measured. After the daily session, the rat was dried under a fan heater and returned to the home cage. All tests were performed from 15:00 to 18:00 hours. The swim path of a rat during each trial was recorded by a video camera (TOTA-450III, TOTA, Japan), and the spatial memory ability was analyzed using the DMS-2 Morris Water Maze system (Institute of Material Medica, Chinese Academy of Medical Sciences, Beijing, China).

4.4. Amino acid neurotransmitter assays

At 30 min, 3 h, 6 h, or 24 h after impulse noise exposure, eight hippocampi in right hemicerebrum per time point were isolated immediately after the animal was sacrificed, and the concentrations of amino acid neurotransmitters, including Glu, GABA, Asp and Gly, were measured using an HPLC system (600E Liquid Chromatography, Waters). The HPLC system was equipped with a reverse-phase column (C18, 150 mm × 4.6 mm, 5 μm; Tridimensional Chromatography, Tianjin, China), coupled with an electrochemical detector. Hippocampal tissue was homogenized in 0.1 M perchloric acid containing dihydroxy-benzylamine (DHBA), and centrifuged at 11,166 g for 20 min at 4 °C. The supernatant affiliated with NaHCO3 and dinitrofluorobenzene (DNFB) was placed in a water bath at 35 °C, and the detection wavelength was 360 nm.

4.5. Immunoblot analysis of NR2B and p-Tau (Thr205)

For immunoblot analysis, six hippocampi in left hemicerebrum per time point (30 min, 3 h, 6 h, or 24 h after impulse noise exposure) were dissected immediately after the animal was sacrificed, and homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 1% Triton X-100, 0.2 mM PMSF, and 1 mM EDTA. Homogenates were centrifuged at 12,000 g for 10 min at 4 °C. The supernatants obtained were immediately placed in boiling water for 10 min. Samples (20 μg protein/lane) were separated on 10% SDS–PAGE gels and electrophoretically transferred to nitrocellulose membranes. Membranes were probed with a rabbit polyclonal antibody to NR2B (1:500, Boster, China), or an anti-p-Tau antibody (1:1000, Bioworld Technology, USA) that recognizes tau phosphorylated at Thr205, or mouse anti-GAPDH monoclonal antibody (1:1000, Santa Cruz, USA), which was used as an internal reference standard. Blots were immunostained using a peroxidase labeled anti-rabbit or anti-mouse secondary antibody (1:1000) and visualized by enhanced chemiluminescence (ECL). Integrated light intensity values from immunoreactive signals were analyzed with the analysis software Gel-Pro 3.1 (Media Cybernetics, USA).

4.6. Tissue preparation for immunohistochemistry

For histology, groups of six rats were anesthetized with pentobarbital sodium 30 min, 3 h, 6 h, or 24 h after impulse noise exposure, and then perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and post-fixed in the same fixative for 48 h, then dehydrated in a graded alcohol series (70%, 80%, 95%, 100%). The tissues were then treated with xylene for 1 h with three changes and immersed in paraffin wax for one and half hours at 56–58 °C with two changes. The paraffin blocks were trimmed and the sections were cut at 8 μm using a microtome according to The Rat Brain in Stereotaxic Coordinates ( Paxinos and Watson, 2007). Coronal sections were then mounted onto polylysine-coated glass slides. Before immunohistochemistry, paraffin sections were deparaffinized in xylene (2 or 3 changes) and hydrated in alcohol:water (100%, 95%, 70% alcohol), then rinsed in distilled water.

4.7. Immunohistochemistry for p-Tau (Thr205) and image data analysis

The deparaffinized sections were incubated in a moist chamber and treated with solutions in the following sequence (the number of repeated steps is given in square brackets): 0.3% H2O2 for 5 min, 0.05 M Tris-buffered saline (TBS, pH 7.4) containing 0.1% Triton X-100 for 5 min [x3], TBS with 10% horse serum for 10 min, TBS containing 1% bovine serum albumin (BSA) and anti-p-Tau (Thr205) for 2 h at 37 °C then overnight at 4 °C, TBS for 5 min [x3], anti-rabbit Bio-IgG diluted 1/150 in TBS for 45 min at 37 °C, TBS for 5 min [x3], HRP-SA diluted 1/200 in TBS for 45 min at 37 °C, TBS for 5 min [x3], DAB stain for 10 min, distilled water, hematoxylin staining for 30 s, and a final rinse in distilled water. Stained sections were then covered with resin before microscopic observations (Olympus DP71, Japan). Immunohistochemical sections were analyzed quantitatively on 3 slices per rat. Region of interest (ROI) was taken from matched littermate pair of sections, and the mean optical density (MOD) for each ROI was determined using image analysis system (Image-Pro Plus 5.0, Media Cybernetics, USA).

4.8. Statistical analyses

All data were analyzed with SPSS 13.0 (SPSS Inc., USA). A one-way ANOVA was used to determine the statistical significance. The level of statistical significance was set at p<0.05 for all tests. Data presented in graphs are group means±SD.

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REFERENCES


