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The nicotinic cholinergic system is affected in rats with delayed carbon monoxide encephalopathy

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HIGHLIGHTS

- We made delayed CO encephalopathy rat model 21days after CO exposure.
- Latencies in the passive-avoidance test were significantly shorter.
- Neuronal cells in the hippocampus were significantly decreased.
- The mRNA expression of *Chrna3* in the hippocampus was significantly decreased and the expression of *Chrna7* in the cerebellum was significantly increased.

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ABSTRACT

Delayed carbon monoxide (CO) encephalopathy may occur following recovery from acute CO poisoning. However, the mechanism of delayed neuronal injury remains unknown. The nicotinic acetylcholine receptors (nAChRs) have been suggested to play a role in cognitive status in neurodegenerative diseases, including Alzheimer's disease. Therefore, in the current study, we investigated the effect of delayed neuronal CO poisoning on gene expression of nAChRs in the hippocampus of Wistar rats. Behavioral effects (measured by the passive-avoidance test) and histological analyses (hematoxylin–eosin-stained hippocampal cell counts and cell death observations) were also investigated, 21 days after CO exposure for 1 h (1000 ppm for 40 min + 3000 ppm for 20 min). Our findings show cognitive impairment and hippocampal cell death, suggesting our rat model is suitable for studying delayed CO encephalopathy. Expression of nAChR (*Chrna3*, *Chrna4*, *Chnra7*, and *Chrnb2*) mRNA was assessed using quantitative real-time polymerase chain reaction. Hippocampal *Chrna3* expression was significantly decreased, and cerebellar *Chrna7* expression significantly increased, in the delayed CO encephalopathy rat model. Thus, the nicotinic cholinergic system may be affected in delayed CO encephalopathy.

1. Introduction

Carbon monoxide (CO) is a colorless, tasteless, non-irritant toxic gas, which is easily absorbed, and by interacting with hemoglobin in red blood cells, leads to carboxyhemoglobin formation. The affinity of hemoglobin for CO is 210 times higher than oxygen [17]. CO poisoning frequently occurs after sufficient inhalation. During acute CO poisoning, i.e. exposure to 1000–3000 ppm (parts per million) CO for 1 h causes headaches, dyspnea and collapse on exertion, nausea, and confusion [4]. CO exposure over 3000 ppm for 1 h causes unconsciousness, coma, and death [4]. Following acute CO exposure, delayed CO encephalopathy may occur after an asymptomatic period of approximately 4 weeks. Ten to thirty percent of patients with this delayed response exhibit personality changes, mild cognitive deficits to severe dementia, psychosis, parkinsonism, and emotional incontinence [2,14]. The mechanisms of delayed CO

Abbreviations: nAChR, nicotinic acetylcholine receptor; AD, Alzheimer's disease; DG, dentate gyrus.

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encephalopathy may include: (1) delayed neuronal death and cholinergic neuronal dysfunction [15]; (2) time-dependent changes of lipid peroxidation and antioxidative status [23]; (3) degradation of myelin basic protein; and (4) sequential activation of the immune system including inflammatory cell invasion [21]. However, the detailed mechanisms of delayed CO poisoning still remain elusive. Brain nicotinic acetylcholine receptors (nAChRs) are a class of ligand-gated channels composed of a number of subunits (α2–10 and β2–4). They can form pentameric channels with two non-identical acetylcholine (ACh) binding sites at the interface between α and β subunits e.g., α4β2, or homomeric channels with five identical ACh binding sites e.g., α7 subunits [3,6].

Nicotinic cholinergic mechanisms play an important role in cognitive function [6]. Thus, we hypothesized that nAChR changes may be implicated in delayed CO encephalopathy, and therefore investigated the role of nAChRs in a rat model of delayed CO encephalopathy.

2. Materials and methods

2.1. Experimental animals

All experiments were conducted in accordance with the Guidelines for Animal Experimentation of Ehime University Graduate School of Medicine (Ehime, Japan). Male Wistar rats (6 weeks old, 132–157 g) from CLEA Japan (Tokyo, Japan) were housed in air-conditioned rooms (temperature, 22 ± 2 °C) set to a 12 h light–dark cycle.

2.2. CO exposure to rats in the delayed CO model

CO exposure was performed as previously described by Thom et al. [21]. Briefly, rats were exposed to 1000 ppm CO for 40 min followed by 3000 ppm for 20 min in a 7.6-L chamber (250 × 190 × 160 mm) (KN-1010-L, Natsume Seisakusho, Tokyo, Japan), until loss of consciousness. We monitored the concentration of CO, CO_2, and O_2 within experiments. Rats were then moved to an air-conditioned room to regain consciousness. Control rats did not receive CO but were exposed to room air for 1 h.

2.3. Passive-avoidance test

Learning and memory function in CO-exposed rats was evaluated using the step-through passive-avoidance test, as in previous studies [15,23]. The apparatus consisted of illuminated (450 × 270 × 260 mm) and dark (450 × 270 × 260 mm) compartments on a grid floor, separated by a sliding door (MPB-M001; Melquest, Toyama, Japan). In the training session before CO exposure, each rat was placed in the illuminated compartment and allowed to explore for 20 s. The sliding door was then opened, and the step-through latency for rats to enter the dark compartment with all four paws was measured. The door was immediately closed upon entry into the dark compartment. At least two additional trials were conducted, each separated by 5 min, until rats entered the dark compartment within 20 s as the same as the previous study [20]. After the door was closed in the last trial, an inescapable foot-shock (1 mA for 5 s) was delivered through the grid floor with a constant current shock generator (SG-100; Melquest, Toyama, Japan). Rats entered the dark compartment within 300 s cut-off latency in the training session, and all tested rats were given foot-shocks until they stopped entering the dark room within this time frame. Rats were treated with or without CO exposure when step-through latencies were >300 s and measured without foot-shocks 24 h after the training session. The step-through latency was recorded as "300 s" when rats did not enter the dark room more than 300 s.

2.4. Histological analysis by hippocampal neuronal cell counts

Three weeks after CO exposure, rats were anesthetized using diethyl ether followed by an intraperitoneal injection of 3% chloral hydrate (1 mL/100 g body weight). Rats were then transcardially perfused with 0.1 M phosphate buffer, followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were subsequently removed, immersed in the same fixative for 5 h at 4 °C and processed for paraffin embedding. Sections (7 μm thick, Bregma −3.3 mm) were dewaxed and rehydrated. Hematoxylin–eosin (HE) staining was performed to determine the effect of CO exposure on the center of CA1, CA3, and the upper blade of dentate gyrus (DG) regions of the hippocampus. We counted cells in these regions for both CO and control groups using a previously reported method [12]. Cell counting was performed blinded with respect to the experimental group.

2.5. Real-time polymerase chain reaction (PCR)

Gene expression of nAChRs was examined 21 days after CO exposure. For gene expression comparisons, we used that one region was selected in one animal randomly and the same region was dissected in the other animals. Rats were sacrificed by decapitation, and brain tissues from nine regions (frontal cortex, temporal cortex, striatum, thalamus, hippocampus, midbrain, pons, cerebellum, and olfactory bulb) bilaterally dissected on an ice-cold stage, according to Glowinski and Iversen [5]. Fresh tissues were weighed and stored at −80 °C until use. RNA was extracted from each brain tissue using the RNeasy kit, including RNase-Free DNase treatment (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. RNA quantity was validated using the Nanodrop1000 (Thermo Scientific, Waltham, MA, USA). RNA integrity was validated using Agilent 2100 Bioanalyzer (Agilent Technologies, Loveland, CO, USA). Each total RNA fraction was reverse transcribed with random hexamers using Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Austin, TX, USA), according to the manufacturer's instructions. TaqMan primer–probe sets for the nAChR subunits, α3 (*Chrna3*) (Rn00583820_m1), α4 (*Chrna4*) (Rn00577436_m1), α7 (*Chrna7*) (Rn00563223_m1), and β2 (*Chrnb2*) (Mm00515323_m1), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, 4352338E) as an endogenous control, were purchased from Applied Biosystems. Real-time quantitative PCR was performed using the StepOnePlus System (Applied Biosystems). Changes in mRNA expression levels (fold changes relative to control) were calculated after *Gapdh* normalization. The calibrator sample was a matched brain region cDNA from a randomly selected control rat. The ΔΔCT method provides a relative quantification ratio according to the calibrator, allowing statistical comparison of gene expression among samples [16]. Values from CO and control groups represent averages of triplicate measurements for each sample.

2.6. Statistical analysis

All values are expressed as mean ± SEM. Repeated measures analysis of variance followed by Dunnett's multiple comparison post hoc test was used to determine the significance of step-through latencies between CO and control groups. Student's *t*-test was used to analyze the significance of cell counts and nAChR mRNA expression between CO and control groups. Statistical analyses were performed using SPSS-J 11.5 statistical software (Chicago, IL, USA). Significance was determined by P values <0.05.