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Research article

Early exposure to urethane anesthesia: Effects on neuronal activity in the piriform cortex of the developing brain

Riichi Kajiwara [a], Ichiro Takashima [b,*]

[a] Department of Electronics and Bioinformatics, Meiji University, Kawasaki, Japan
[b] National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan

HIGHLIGHTS

- Early exposure to urethane anesthesia impairs piriform cortex function.
- Short-interval intra-cortical inhibition is attenuated.
- The current sink in layer Ib remains active through successive stimulation of the LOT.
- Optical imaging reveals stronger and extended neural activity in the impaired cortex.
- GABA-immunoreactive cell density is reduced.

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ABSTRACT

Exposure to urethane anesthesia reportedly produces selective neuronal cell loss in the piriform cortex of young brains; however, resulting functional deficits have not been investigated. The present study found abnormalities in piriform cortex activity of isolated brains *in vitro* that were harvested from guinea pigs exposed to urethane anesthesia at 14 days of age. Current source density (CSD) analysis and voltage-sensitive dye (VSD) imaging experiments were conducted 48 h after urethane injection. We applied paired-pulse stimulation to the lateral olfactory tract (LOT) and assessed short-interval intracortical inhibition in the piriform cortex. CSD analysis revealed that a current sink in layer Ib remained active in response to successive stimuli, with an inter-stimulus interval of 30–60 ms, which was typically strongly inhibited. VSD imaging demonstrated stronger and extended neural activity in the urethane-treated piriform cortex, even in response to a second stimulus delivered in short succession. We identified gamma-aminobutyric acid (GABA) ergic neurons in the piriform cortex of sham and urethane-treated animals and found a decrease in GABA-immunoreactive cell density in the urethane group. These results suggest that urethane exposure induces loss of GABAergic interneurons and a subsequent reduction in paired-pulse inhibition in the immature piriform cortex.

1. Introduction

Anesthetic-induced developmental neurotoxicity is well known. General anesthetics have *N*-methyl-D-aspartate (NMDA) receptor-blocking or γ-aminobutyric acid (GABA) receptor-activating properties; a number of animal studies indicated that exposure to NMDA antagonists and GABA agonists causes widespread neuronal apoptosis in the immature brain [1]. For example, ethanol reportedly induced apoptotic neurodegeneration in the developing rat brain, with the most affected regions being the parietal, cingulate and frontal cortices, as well as the thalamus [2]. Interestingly, Thompson and Wasterlain reported that urethane anesthesia produced selective damage to the piriform cortex [3]. According to their study, neuronal cell loss was most severe in 2-week-old rats 48 h after urethane injection. Whether specific anesthetic agents lead to damage in specific brain regions depending on the timing of administration is of great interest. Such an animal model would be useful for studying the functional development of the target brain region. Although Thompson and Wasterlain reported urethane-induced piriform cortex damage, the consequences of this damage on piriform cortex function has not been determined.

The aim of this study was to examine piriform cortex functional impairment following early exposure to urethane anesthesia. We

* Corresponding author. Fax: +81 29 861 5849.
E-mail address: i.takashima@aist.go.jp (I. Takashima).

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generated a urethane-damaged animal model according to the procedure used by Thompson and Wasterlain, but used young guinea pigs instead of rats. Until now, no evidence existed suggesting that urethane exposure caused damage to guinea pig brains similar to that seen in rats. Even if urethane anesthesia was damaging to the guinea pig brain, the most vulnerable period for guinea pigs may differ from that of rats, as the timing of the brain growth spurt differs between the species [4]. Nevertheless, for our initial study, guinea pigs were used because we employed an *in vitro* isolated whole-brain preparation, which was established using guinea pig brains [5], to evaluate the piriform cortex activity in an intact neural network. By applying current source density (CSD) analysis and voltage-sensitive dye (VSD) imaging to the piriform cortices harvested from urethane-experienced animals, we showed here abnormal neural activity in response to paired-pulse stimulation to the lateral olfactory tract (LOT). Changes in piriform cortex GABA-immunoreactive neuronal density were also examined.

2. Material and methods

2.1. Animals

All of the animal procedures were reviewed and approved by the Committee on Animal Care and Use and by the Ethical Committee of the National Institute of Advanced Industrial Science and Technology.

Young guinea pigs (180–200 g, 14 days old) were injected intraperitoneally (i.p.) with urethane (1.25 g/kg in saline, $n = 12$) or saline ($n = 12$). The urethane-injected animals were allowed to wake up from anesthesia, usually within 5–7 h. At 48 h after the injection of urethane or saline, 16 animals were used for *in vitro* whole-brain experiments (urethane: $n = 8$, saline: $n = 8$), and the remaining eight animals were used for immunohistochemistry (urethane: $n = 4$, saline: $n = 4$). During the 48 h, the animals were able to obtain nourishment for themselves, and no body weight loss was observed even in the urethane-treated animals. All animals contributed to their respective analyses (*i.e.*, no animals were excluded).

2.2. Isolated whole-brain preparation

Animal brains were dissected out according to the standard procedure [5–8] after anesthesia with pentobarbital (60 mg/kg, i.p.). A solution containing (in mM) 126 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4$CaCl_2$, 26 $NaHCO_3$, 15 glucose, 2.1HEPES, and 3% dextran [M.W. 70,000], oxygenated with a 95% O_2/5% CO_2 gas mixture was artery perfused at 6.5 mL/min. Experiments were performed at 32 °C. The ventral view of the isolated guinea pig brain is shown in Fig. 1A.

2.3. Electrophysiological recordings

Field potential laminar profiles were recorded utilizing 16-channel silicon multiprobes (impedance 2–3 MΩ, 100-μm separation between recording sites) (NeuroNexus Technologies, USA). The multiprobe was inserted perpendicular to the pial surface of the anterior piriform cortex (APC). Field signals were amplified and band-pass filtered between 0.1 Hz and 3 kHz (HST/16o25 headstage, 32-channel preamplifier box; Plexon Inc., USA), and digitized at 10 kHz. Bipolar tungsten electrodes with a tip separation of 150 μm (FHC, USA) were positioned on the LOT for stimulation. The right panel of Fig. 1A shows the arrangement of the stimulation and recording electrodes.

By applying single-pulse stimulation (10–100 μA; 200 μs) to the LOT, we first determined a stimulus intensity that produced a half-maximum response for use in subsequent paired-pulse experiments. To assess intracortical inhibition in the APC [6,7], paired stimulation was delivered with a 30–250-ms inter-pulse interval, and then an average of 10 responses was utilized to build field potential laminar profiles. We used CSD analysis to identify the pattern of current sources and sinks from the field potential profiles.

2.4. Optical imaging

Optical recordings with a voltage-sensitive dye were performed according to a previously described technique [7–9]. The brain was stained for 25 min with 1.5 mL of the voltage-sensitive dye solution, containing 0.2 mM Di-4-ANEPPS (Invitrogen, USA) in 2.5% ethanol, 0.13% Cremaphor EL (Sigma–Aldrich, USA), 1.17% distilled water, 48.1% fetal bovine serum (FBS; Sigma–Aldrich), and 48.1% of the abovementioned perfusate solution. Epifluorescence measurements were accomplished using a MiCAM02-CMOS high-speed camera system (BrainVision, Japan) mounted on a fluorescence macro zoom microscope (MVX-10, Olympus, Japan). Optical responses were presented as 92 × 80 pixel images at a rate of 1.0 ms/frame, covering a cortical area of 2.4 × 2.0 mm. The camera was positioned to cover the most posterior region of the APC. For each optical image shown, eight responses were averaged.

2.5. Immunohistochemistry

Guinea pigs used for immunohistochemical experiments were deeply anesthetized with pentobarbital and transcardially perfused with a fixative containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed and cut into blocks containing the APC; the blocks were then immersed in 30% sucrose in PB for 2 days. Series of 40-μm-thick coronal sections were cut on a freezing microtome, and every seventh section was processed for GABA immunocytochemistry using the avidin-biotin technique [10]. Sections were washed thoroughly in 0.01 M phosphate buffered saline (PBS; pH 7.5) and incubated in 0.5% H_2O_2 for 30 min to block endogenous peroxidase activity. After three rinses in PBS, the sections were preincubated in 1.5% normal horse serum (NHS)/2% bovine serum albumin (BSA) and 0.3% Triton-X 100 for 60 min and transferred to a solution containing a monoclonal mouse antibody against GABA (1:1,000; A0310, Sigma–Aldrich), 0.75% NHS, and 1% BSA for 24 h at 4 °C. The sections were washed three times in PBS, reacted for 60 min with the secondary biotinylated anti-mouse immunoglobulin (Ig)G antibody (Vectastain Elite ABC Kit, PK-6102, Vector Laboratories Ltd., USA) diluted 1:200 in PBS and then washed three times in PBS. Finally, the sections were incubated with avidin–biotin–horseradish peroxidase complex (ABC-HRP) for 60 min, washed three times in PBS, and then subjected to the nickel-intensified diaminobenzidine (DAB) reaction in the presence of 0.01% H_2O_2. A standard 5-min DAB reaction time was used for all brain slices from both groups.

2.6. Cell counting

Immunohistochemical sections were imaged using light microscopy and computer-assisted image capturing. Cell counting was performed using Corel Photo-Paint software (Corel, Canada). The number of GABA-immunoreactive neurons was determined in one visual field (500 μm in diameter) per section. The center of the visual field was always positioned in the middle of APC layer II. Cells with round or oval somata and a diameter of 10–15 μm were counted [10]. To avoid overestimation of the cell number due to cell fractionation, cell counts were corrected using the Abercrombie method [11]. These measurements were performed in 12 corresponding sections per animal, covering the APC where opti-