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Only extra-high dose of ketamine affects L-glutamate-induced intracellular Ca^{2+} elevation and neurotoxicity☆

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ABSTRACT

The neurotoxic effects of anesthetics on the developing brain are a concern. Although most of the anesthetics are $GABA_A$ agonists or NMDA antagonists, the differences in these effects on prospective glutamate-neurotoxicity in the brain is not fully understood. We examined the degree of L-glutamate-induced intracellular calcium ($[Ca^{2+}]i$) elevation and neurotoxicity in neurons exposed to anesthetics. Primary cortical neurons from E17 rats were preincubated with 1–100 μM of ketamine or thiopental sodium (TPS) for the first 72 h of culturing. Two weeks later, the neurons were exposed to L-glutamate. The extent of glutamate toxicity was evaluated using Ca^{2+}-imaging and morphological experiments. Preincubation with 100 μM ketamine but not with other concentrations of ketamine and TPS for the first 72 h in culture significantly enhanced L-glutamate-induced $[Ca^{2+}]i$ elevation 2 weeks later. Morphology experiments showed that vulnerability to L-glutamate-mediated neurotoxicity was only altered in neurons preincubated with 100 μM ketamine but not with TPS. Although preincubation with high concentration of ketamine showed enhancement of L-glutamate-induced $[Ca^{2+}]i$ elevation 2 weeks later, long-term exposure to TPS or ketamine at clinical doses during developmental periods may not result in a dose-related potentiation of exogenous glutamate-induced neurotoxicity, once the intravenous anesthetics are discontinued.

1. Introduction

It has been reported that commonly used anesthetics can cause neurodegeneration and long-term neurocognitive deficits (Olney et al., 2002; Jevtovic-Todorovic et al., 2003). In particular, neuronal damage induced by ketamine, one of the most commonly used intravenous anesthetic, in the immature brain has been investigated. It is shown that ketamine-induced suppression of neuronal activities leads to neuronal death during the development stages in rodents (Ikonomidou et al., 1999; Zou et al., 2009; Liu et al., 2011; Yan and Jaing (2014)). Prolonged exposure to ketamine, which blocks NMDA receptors continuously, causes cell death in the developing brain by a mechanism involving a compensatory upregulation of NMDA receptor subunits. This upregulation could be associated with toxic accumulation of intracellular calcium, increased oxidative stress, and activation of nuclear factor-kappa B (NF-κB) signaling pathway, and makes neurons more vulnerable even after ketamine is cleared (Wang et al., 2000, 2005, 2006; Liu et al., 2013).

Thiopental sodium (TPS) is also one of the most popularly used anesthetics that act as a gamma-aminobutyric acid (GABA) receptor agonist. In the developing brain, $GABA_A$ receptor agonists as well as NMDA receptor antagonists are suspected to have neurodegenerative effects. These agents are thought to cause a reversal of the chloride gradient, plasma membrane depolarization, and calcium influx, leading to subsequent neurotoxicity and apoptosis (Ben-Ari, 2002; Nunez et al., 2003; Wei, 2011).

Since most of the currently used anesthetics act as either NMDA receptor antagonist or $GABA_A$ agonist, it is important to compare

Abbreviations: 5-FU, 5-fluoro-2′-deoxyuridine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; AR, area ratio; $[Ca^{2+}]i$, intracellular calcium; CCD, cooled charge-coupled device; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; E17, embryonic day 17; FCS, fetal calf serum; Fmax, the mean maximum change in fluorescence intensity; GABA, gamma-aminobutyric acid; GFAP, glial fibrillary acidic protein; HR, height ratio; HS, horse serum; HSD, honestly significance different; NBS, normal bath solution; NF-κB, nuclear factor-kappa B; NMDA, N-methyl-D-aspartate; PBS, phosphate buffer solution; PD, postnatal day; SEM, standard error of the mean; SR, survival ratio; TPS, thiopental sodium.

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the effects of these anesthetic agents on the developing nervous system. At present, there is no data available regarding the right period of administration or safe dosage for anesthetics such as TPS and ketamine, during the neuronal development.

Under physiological conditions, calcium acts as a signaling pathway resulting from activation of ion channels or as a second messenger. Cytosolic calcium is involved in several important biochemical reactions related to synaptic function and gene transcription (Graef et al., 1999). Measurement of intracellular calcium concentration in neurons enables us to determine the neuronal activities related to Ca^{2+} regulations both *in vivo* and *in vitro*. Previously, we examined the intracellular calcium response to extracellular signaling factors, such as acetylcholine and nicotine, with distinct effects on embryonic cells *in vitro* (Sekiguchi-Tonosaki et al., 2009). The failure of calcium regulation is considered to be involved in the neurotoxic effects induced by intravenous anesthetics in the developing brain (Wang et al., 2000; Jevtovic-Todorovic et al., 2003; Sinner et al., 2011; Wei and Inan, 2013).

In the present study, using calcium imaging and morphological examinations *in vitro*, we aimed to determine whether a long time exposure to TPS or ketamine during perinatal stages would result in a dose-related potentiation of exogenous glutamate-induced intracellular calcium elevation and neurotoxicity once the intravenous anesthetics are removed.

2. Materials and methods

2.1. Ethical approval

All animals were treated in strict accordance with NIH and the institutional guidelines for the care and treatment of laboratory animals. The study protocol was approved by the Animal Care Committee at the Osaka University Graduate School of Medicine (Ref: 21-061-2). We do confirm that all efforts were made to minimize the number of animals used and their suffering.

2.2. Chemical reagents

The chemicals used in this study were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM), 4-aminobutyric acid (GABA) and KCl from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); 5-fluoro-2′-deoxyuridine (5-FU), poly-L-lysine, streptomycin, penicillin, pluronic acid F-127, L-glutamate acid, and anti-microtubule-associated protein 2 (MAP2), (±)AMPA from Sigma–Aldrich (St Louis, MO, USA); trypsin from Difco Lab (Detroit, MI); fetal calf serum (FCS) from ICN Biochemicals (Costa Mesa, CA, USA); B-27 Supplement Minus antioxidants (AO) and horse serum (HS) from Gibco BRL (Carlsbad, CA, USA); anti-glial fibrillary acidic protein (GFAP) antibody from Dako (Carpinteria, CA, USA); TPS from Mitsubishi Tanabe Pharma Corp. (Osaka, Japan); Ketamine from Daiichi-Sankyo Company Ltd. (Tokyo, Japan); and Fluo4-AM from Dojindo (Kumamoto, Japan).

2.3. Cell culture

Primary cultures of cortical neurons were prepared as described previously (Shibuta et al., 2000, 2010; Varathan et al., 2001). To summarize, rat fetuses from anesthetized pregnant Wistar rats, which were obtained from Nihon SLC (Hamamatsu, Japan), were removed on embryonic day 17 (E17). The fetal rat brains were examined under a microscope. Cerebral cortical neurons were treated with 0.25% trypsin in phosphate buffer solution (PBS) at 37 °C for 20 min and titrated with a Pasteur pipette. Dispersed cells were diluted to a concentration of $0.6–1.0 \times 10^6$ cells/mL in DMEM, which contained 8% FCS, 4% HS, 2% B-27 Supplement Minus AO, 50 μg/mL streptomycin, and 50 IU/mL penicillin. This suspension was placed in a poly-L-lysine-coated 35 mm diameter film-bottom dish FD10300 (Matsunami Glass Ltd., Osaka, Japan) for Ca imaging experiments or in 2 mm grid tissue culture dishes (1.5 mL per well) (Nunc, Naperville, IL, USA) for the cytotoxicity assay. We used the grid tissue culture dishes to observe the same neurons in a given area over time, as described previously (Shibuta et al., 2000).

During the first 72 h in primary culture, the neurons were exposed to 1, 10, or 100 μM TPS or ketamine, respectively. In the preliminary experiments, we observed that 100 μM ketamine did not elicit $[Ca^{2+}]i$ elevation, while the $[Ca^{2+}]i$ elevation elicited by 100 μM TPS was significantly smaller than that elicited by 1 μM GABA, which did not kill neurons at all on DIV2. In addition, these anesthetics concentrations we used in the present experiments were consistent with the free plasma concentrations (median effective dose: EC_{50}) of ketamine and TPS for general anesthesia (approximately 7 and 25 μM, respectively). Since ketamine at 10 μM would occupy 60% of the available NMDA receptors, these concentrations are compatible with previous studies (Flood and Krasowski, 2000; Tassonyi et al., 2002).

As a control, a couple of culture dishes were exposed in parallel to similar amounts of PBS without an anesthetic. Only one such culture dish, which was not contaminated, was used for data collection and the others were discarded without further inspection.

After 72 h in culture, the medium was fully replaced. The cells were treated with 5 μg/mL of 5-FU for 3 days to prevent the proliferation of non-neuronal cells. The neurons were maintained in DMEM containing 8% FCS, 4% HS, and 2% B27 supplement in an atmosphere consisting of 5% CO_2 and 95% air, 100% humidity, at a temperature of 37 °C. Half of the medium was changed twice weekly, thereafter. All subsequent experiments were carried out after 13–14 days *in vitro* (DIV).

2.4. Immunohistochemical assessment

As previously described (Varathan et al., 2001), in order to confirm the purity of the neuronal cultures, cells were immunostained with anti-MAP2 or anti-GFAP antibody, before and after the experiment. More than 90% expressed MAP2 and less than 5% of the cells expressed GFAP regardless of the duration of the experiments. This demonstrated that most of the cells in our cultures were neurons.

2.5. Calcium imaging

Calcium imaging was performed as previously published (Sekiguchi-Tonosaki et al., 2009). The cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]i$) was measured using a fluorescence measurement system (Aquacosmos®; Hamamatsu Photonics, Hamamatsu, Japan) and an inverted phase contrast microscope (Axiovert 200®, Zeiss). Neurons were transferred to a normal bath solution (NBS; 137 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.3, and 22 mM glucose) containing a Ca^{2+}-sensitive indicator, 10 μM Fluo4-AM, and 0.025% pluronic acid F-127 for 30 min at room temperature.

Cultures were rinsed twice with fresh NBS and placed onto a microscope stage. A 150-W xenon lamp was used for fluorescence excitation (450–490 nm) of neurons, and 16-bit fluorescence images (512×512 pixel; 2×2 binned) were obtained using a cooled charge-coupled device (CCD) camera (Orca ER®; Hamamatsu Photonics) connected to the microscope with an oil-immersion objective lens (Fluor® ×40, oil, numerical aperture 1.30; Zeiss). A series of images was acquired at 2-s intervals for 120 s. During the exposures, excitation light was blocked using a filter exchanger (C8214; Hamamatsu Photonics) to avoid potential cell damage. Images were analyzed using a processing software (Aquacosmos®, Hamamatsu Photonics, Japan).