Int. J. Devl Neuroscience 51 (2016) 42–49

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Continuous monitoring of caspase-3 activation induced by propofol in developing mouse brain

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ARTICLE INFO

Article history:
Received 10 February 2016
Received in revised form 20 April 2016
Available online 26 April 2016

Keywords:
Propofol anesthesia
Caspase-3
FRET
SCAT3
Neonatal mouse

ABSTRACT

The neurotoxicity of anesthetics on the developing brain has drawn the attention of anesthesiologists. Several studies have shown that apoptosis is enhanced by exposure to anesthesia during brain development. Although apoptosis is a physiological developmental step occurring before the maturation of neural networks and the integration of brain function, pathological damage also involves apoptosis. Previous studies have shown that prolonged exposure to anesthetics causes apoptosis. Exactly when the apoptotic cascade starts in the brain remains uncertain. If it starts during the early stage of anesthesia, even short-term anesthesia could harm the brain. Therefore, apoptogenesis should be continuously monitored to elucidate when the apoptotic cascade is triggered by anesthesia. Here, we describe the development of a continuous monitoring system to detect caspase-3 activation using an in vivo model. Brain slices from postnatal days 0–4 SCAT3 transgenic mice with a heterozygous genotype ($n = 20$) were used for the monitoring of caspase-3 cleavage. SCAT3 is a fusion protein of ECFP and Venus connected by a caspase-3 cleavable peptide, DEVD. A specimen from the hippocampal CA1 sector was mounted on a confocal laser microscope and was continuously superfused with artificial cerebrospinal fluid, propofol (2,6-diisopropylphenol, 1 μM or 10 μM), and dimethyl sulfoxide. Images were obtained every hour for five hours. A pixel analysis of the ECFP/Venus ratio images was performed using a histogram showing the number of pixels with each ratio. In the histogram of the ECFP/Venus ratio, an area with a ratio > 1 indicated the number of pixels from caspase-3-activated CA1 neurons. We observed a shift in the histogram toward the right over time, indicating caspase-3 activation. This right-ward shift dramatically changed at five hours in the propofol 1 μM and 10 μM groups and was obviously different from that in the control group. Thus, real-time fluorescence energy transfer (FRET) imaging was capable of identifying the onset of apoptosis triggered by propofol in neonatal brain slices. This model may be a useful tool for monitoring apoptogenesis in the developing brain.

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1. Introduction

Exposure to some anesthetics and sedative drugs can reportedly have a damaging effect on the developing brain. Such neurotoxicity has been confirmed histopathologically, presenting as apoptosis in the developing brain. Previous reports have demonstrated that apoptotic changes occur several hours after exposure to anesthetics (Brambrink et al., 2010; Istaphanous et al., 2011; Satomoto et al., 2009; Zou et al., 2011). Creeley et al. confirmed the presence of obvious histopathological changes, suggesting the apoptosis of neurons and oligodendrocytes, at three hours after exposure to propofol for five hours (Creeley et al., 2013). The late appearance of apoptosis has also been confirmed in neuronal cultures. Pearn et al. (2012) confirmed the presence of apoptosis at six hours after exposure to propofol in a primary neuronal culture. However, all of these previous studies confirmed the presence of apoptosis histopathologically, based on the appearance of neuron shrinkage or TUNEL-positive neurons or Western blotting findings for cleaved caspase; these methods visualize the consequences of apoptosis,

Abbreviations: FRET, fluorescence energy transfer; ACSF, artificial cerebrospinal fluid; TNFα, tumor necrosis factor-α; CHX, cycloheximide; DMSO, dimethyl sulfoxide.

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http://dx.doi.org/10.1016/j.ijdevneu.2016.04.007
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not the actual process of apoptosis. Information on when apoptosis is initiated after exposure to anesthetics and also the ability to differentiate normal physiological apoptosis in the developing brain and pathological apoptosis induced by drugs are needed. Determining whether apoptosis induced by anesthetics requires a prolonged exposure or if the switch to the apoptotic cascade commences at an early stage and may require a long time to reach the final execution of apoptosis is also important. Such information would help us to determine whether short periods of exposure to anesthetics are safe for neonatal and fetal brains.

Fluorescence energy transfer (FRET) technology can provide real-time information about the dynamics and pattern of endogenous enzyme activation in vivo (Takemoto et al., 2003;Tyas et al., 2000). Briefly, FRET is based on the phenomenon of distance-dependent energy transfer from a donor molecule to an acceptor molecule. If the donor and acceptor are linked by a molecular sequence that is cleaved by the action of an enzyme, the FRET disappears when the linker is cleaved. Using this phenomenon, the cleavage of linkers can be monitored by observing changes in the wavelengths of the emissions. SCAT3 is a probe for detecting caspase cleavage that utilizes ECFP and Venus as a fluorescent donor and acceptor, respectively. Previous studies have shown that this probe can actually detect the real-time cleavage of caspase-3 in transgenic mice and transfected cells (Nakazawa et al., 2013;Yamaguchi et al., 2011). Thus, this biosensor was expected to be useful for monitoring the activation of caspase-3 in response to anesthetic exposure in vivo. Here, we describe a real-time imaging system for monitoring caspase-3 activation in mouse brain slices to identify the initiation of apoptosis.

2. Materials and methods

2.1. Animal care and use

Postnatal day (PND) 0-4C57BL/6J mice were used (n = 20). Animal care and all the experimental procedures were approved by the Institutional Animal Research Committee of Showa University and were in accordance with Japanese Government Law No. 105. All efforts were made to minimize the number of animals to be used and their suffering.

2.2. SCAT3 transgene

SCAT3 is a fluorescence resonance energy transfer (FRET) probe that consists of a donor (enhanced cyan fluorescent protein [ECFP]) and an acceptor (Venus, a mutant of yellow fluorescent protein). The donor and the acceptor are linked with a caspase-3 recognition and cleavage sequence (DEVD). With this probe, activated caspase-3 cleaves the linker peptide (DEVD) and abolishes the FRET, resulting in a marked increase in the lifetime of ECFP. Therefore, the dissociation of ECFP and Venus upon cleavage of the linker increases the ECFP/Venus ratio.

The EcoRI fragment of pJC13-1 was removed and replaced with a NotI site, and the BamHI fragment of pJC13-1 was then removed and replaced with the SCAT3 expression cassette (CAG-SCAT3-pA) to create a 2 × HS4-CAG-SCAT3-pA-2 × HS vector.

HeLa cells were plated on polyethyleneimine-coated glass coverslips and were transfected with 0.5 μg of plasmid vector for 6 h, then maintained in growth medium for another 12–18 h.

A transgenic mouse strain with the caspase3 active detection indicator SCAT3 (Takemoto et al., 2003) under CAG promoter control (Yamaguchi et al., 2011) was used. This mouse strain was provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan.

2.3. HeLa cell culture and imaging analysis

HeLa cells were maintained in DME (Sigma-Aldrich) supplemented with 10% FBS. Transfections of the HeLa cells were performed using the SuperFect reagent (QIAGEN). SCAT3-expressing HeLa cells were exposed to 50 ng/mL of tumor necrosis factor-α (TNFα) and 10 μg/mL of cycloheximide (CHX) for three hours at room temperature.

2.4. Preparation of slices

The experiments were performed with brain slices from postnatal days (P) 0-4 SCAT3 transgenic mice with a heterozygous genotype (n = 20). The day of birth was defined as P0. The animals were anesthetized deeply with isoflurane and then decapitated. Each brain was removed rapidly and placed in ice-cold (4 °C) artificial cerebrospinal fluid (ACSF) containing the following (in mM): 130 NaCl, 3 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 10 glucose. The ACSF was continuously bubbled with a 95% O_2-5% CO_2 gas mixture to establish a pH of 7.4. The brain was cut into 350- to 400-μm coronal sections that included the hippocampal CA1 region using a vibrating microslicer (VT1200S; Leica Microsystems Japan, Tokyo, Japan). The slices were allowed to recover in a holding chamber containing ACSF at 34 °C for one hour and then were maintained at room temperature (22–25 °C) in ACSF.

2.5. Administration of agents and observation

Twenty slices from twenty P0-4 animals were transferred into a recording chamber that was mounted on a confocal laser microscope (A1R; Nikon, Tokyo, Japan) equipped with a water immersion × 40 (0.8 NA) objective (Nikon Instruments), and the slices were superfused continuously with ACSF at a rate of 2.0 mL/min at 37 °C using a peristaltic pump and a warming device (Thermo Plate; Warner Instruments, Hamden, CT, USA). Propofol (2,6-diisopropylphenol; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in an organic solvent (dimethyl sulfoxide [DMSO]; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and applied to the ACSF at a final concentration of 1 μM or 10 μM at 37 °C. DMSO without propofol was added to the ACSF in the DMSO group.

SCAT3 is a fusion protein of ECFP and Venus connected by a caspase-3 cleavable peptide, DEVD. Cleavage of DEVD impairs the intracellular fluorescence resonance energy transfer (FRET) between the two fluorescent proteins and changes the emission wavelength from 525 nm for Venus to 475 nm for ECFP. We used a 457.9-nm excitation filter, a 465- to 500-nm emission filter (ECFP), and a 525- to 555-nm emission filter (Venus). The wavelength changes in CA1 neurons expressing SCAT3 were captured using an imaging scanner and were imaged at a frame rate of 4 Hz using an image acquisition and analysis system (NIS-Elements, Nikon Instruments). We used imaging software (NIS-Elements, Nikon, Tokyo, Japan), and the hippocampal CA1 neurons were scanned in region of interest (ROI)-integrated Z-axis images of all the layers. Caspase-3-activated cells were evaluated as pseudocolor changes from blue to red on the ECFP/Venus ratio images. The z stacks (5–8 μm) and time-lapse images of the hippocampal CA1 neurons were recorded every hour for five hours.

To visualize the living hippocampal CA1 neurons from SCAT3 transgenic neonatal mice, we set up a live-imaging system using a fast-scanning confocal microscope, which allowed us to scan the slice regions within a short time and to reduce photobleaching and phototoxicity as much as possible.

A pixel analysis of the ECFP/Venus ratio images was performed using a histogram showing the number of pixels with each ratio. In the histogram of the ECFP/Venus ratio, an area with a ratio > 1 indicated the number of pixels from caspase-3-activated CA1 neurons.

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