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ANESTHESIA INDUCES NEURONAL CELL DEATH IN THE DEVELOPING RAT BRAIN VIA THE INTRINSIC AND EXTRINSIC APOPTOTIC PATHWAYS

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Abstract—It was shown recently that exposure of the developing rat brain during the peak of synaptogenesis to commonly used general anesthetics can trigger widespread apoptotic neurodegeneration in many regions of the developing rat brain and persistent learning/memory deficits later on in life. To understand the mechanism by which general anesthetics induce apoptotic neuronal death we studied two common apoptotic pathways—the intrinsic and the extrinsic pathway—at different time points during synaptogenesis. We found that the intrinsic pathway is activated early on during anesthesia exposure (within two hours), as measured by the down-regulation of bcl-x_L, up-regulation of cytochrome c and the activation of caspase-9 in 7-day-old rats (the peak of synaptogenesis), but remains inactivated in 14-day-old rats (the end of synaptogenesis). The extrinsic pathway is activated later on (within six hours of anesthesia exposure), as measured by the up-regulation of Fas protein and the activation of caspase-8 in 7-day-old rats, but remains inactivated in 14-day-old rats. Anesthesia-induced apoptotic neurodegeneration is age dependent with vulnerability closely correlating with the timing of synaptogenesis, i.e. the developing brain is most sensitive at the peak of synaptogenesis (7 days old) and least sensitive at the end of synaptogenesis (14 days old).

Key words: immature brain, synaptogenesis, apoptosis, caspase, bcl-2, cytochrome c.

The brain growth spurt period (i.e. synaptogenesis) happens at different times in different species. In humans, it is both a pre- and postnatal phenomenon (from the 6th month of gestation to a couple of years after birth), and in rats it is mainly a postnatal phenomenon (first two weeks of life) (Dobbing and Sands, 1979). During normal synaptogenesis, programmed cell death (i.e. apoptosis) occurs, which enables the elimination of a very small percentage of neurons (less than 1%) that are considered redundant.

We have recently reported that the volatile anesthetic isoflurane, alone or in combination with midazolam (a benzodiazepine i.v. anesthetic) and nitrous oxide (N_2O) (an inhalational anesthetic), when used to maintain a surgical plane of anesthesia for 6 h in the immature rats that were at the peak of synaptogenesis (7 days old), triggered widespread apoptotic neurodegeneration in many regions of the developing brain. Moreover, when rats, exposed to these anesthetics in infancy, were tested as juveniles and adults, they were found to have persistent learning/memory deficits (Jevtovic-Todorovic et al., 2003).

Although it is known that the developing rat brain is extremely sensitive to the apoptogenic action of common general anesthetics at the peak of synaptogenesis (7 days of age), it is not clear how the vulnerability of the immature neurons coincides with the timing of brain development. To address this issue we exposed immature rats of different ages (from 1 to 14 days of age) to three common general anesthetics, isoflurane, N_2O and midazolam, alone or in clinically relevant combination, and analyzed the severity and distribution of apoptotic neurodegeneration in order to determine whether the peak of vulnerability coincides with the peak of synaptogenesis.

Apoptosis can be executed via different biochemical pathways—an intrinsic and an extrinsic apoptotic pathway. They both result in activation of effector caspases as the final step (Meier et al., 2000; Hengartner, 2000). The intrinsic apoptotic pathway involves the down-regulation of anti-apoptotic proteins from the bcl-2 family (e.g. bcl-x_L), an increase in mitochondrial membrane permeability and an increased release of cytochrome c into the cytoplasm, which in turn activates caspase-9 and caspase-3 resulting in apoptotic damage (Hengartner, 2000). The extrinsic pathway is initiated by the activation of death receptors that involves the formation of a death-inducing signaling complex (DISC), which contains Fas, a member of the TNF-α superfamily. DISC formation results in the activation of caspase-8, which activates caspase-3, executing the cell (Chinnaiyan et al., 1995; Medema et al., 1997). It is not established which apoptotic pathway gets activated during anesthesia exposure.

To investigate the importance and the timing of the intrinsic pathway activation in the anesthesia-induced neuroapoptosis, we analyzed the changes in the expression of

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Abbreviations: AD, anterodorsal thalamic nucleus; AM, anteromedial thalamic nucleus; AV, anteroventral thalamic nucleus; cCBF, cortico-cerebral blood flow; DAB, 3,3′-diaminobenzidine; DISC, death-inducing signaling complex; DMSO, dimethyl sulfoxide; ICC, immunocytochemistry/immunochemical staining; LD, laterodorsal thalamic nucleus; MAC, minimum alveolar anesthetic concentration that prevents purposeful movement to supramaximal noxious stimulation in 50% subjects; NMDA, *N*-methyl-D-aspartate; NR, nucleus reuniens; N_2O, nitrous oxide; P, postnatal; PBS, phosphate-buffered saline.

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two key proteins: bcl-x_L, and cytochrome c, and the activity of the key effector caspase (caspase-9). To investigate the importance and timing of the extrinsic pathway activation in anesthesia-induced neuroapoptosis, we analyzed the changes in the expression of Fas, and the activity of the key effector caspase (caspase-8). We selected two representative age groups: 7 days of age (postnatal day 7, P7) (at the peak of synaptogenesis) and 14 days of age (at the end of synaptogenesis).

We found that the immature rat brain is most vulnerable in general to anesthesia-induced neuroapoptosis at the peak of synaptogenesis (at P7) and that both the intrinsic and the extrinsic apoptotic pathways are activated by general anesthetics, although the initial apoptotic insult is most likely due to the activation of the intrinsic pathway.

EXPERIMENTAL PROCEDURES

Animals

Sprague–Dawley rat pups of various ages (1-, 3-, 7-, 10- and 14-days of age, the average body weights were approximately 7, 9, 18, 24 and 33 g, respectively) were used for all experiments. At postnatal (P) day of interest (P1, P3, P7, P10 or P14), experimental rats were exposed to 2, 4 or 6 h of anesthesia, and controls were exposed to 2, 4 or 6 h of mock anesthesia. They were randomly divided into three groups. One group was used for cerebral perfusion studies, which involved continuous cortical cerebral blood flow (cCBF) measurements during anesthesia. The second group was used for acute biochemical studies (e.g. Western blotting) of cytochrome c, bcl-x_L and Fas. The third group was allowed to recover and was used for histopathology studies (e.g. caspase-3, -8 and -9 and silver staining) at several post-anesthesia intervals.

Anesthesia treatment

N_2O and oxygen were delivered using a calibrated flowmeter. Midazolam was dissolved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) immediately before administration. For control experiments, 10% DMSO was used. To administer specific concentrations of N_2O, both normobaric [1 atm, 100 vol%] and hyperbaric conditions were required. In experiments requiring hyperbaric conditions, the N_2O/oxygen (GTS, Allentown, PA, USA) mixture was introduced at a pressure of 2.0 atm (200 vol%=150 vol% N_2O and 50 vol% oxygen) and sustained for the duration of the experiment (Jevtovic-Todorovic et al., 1998, 2001). A relief valve on the hyperbaric chamber allowed continuous escape of gases to avoid accumulation of carbon dioxide. Animals were kept normothermic throughout the experiment, as previously described (Jevtovic-Todorovic et al., 2000). For N_2O concentrations of $<$80 vol%, normobaric conditions were used. The N_2O/oxygen mixture was delivered to the anesthesia chamber while a relief valve was kept open so that pressure inside the chamber remained at 1 atm. For control experiments, air (GTS) was substituted for the gas mixtures. For experiments with isoflurane (Abbott, Abbott Park, IL, USA), we used an agent specific vaporizer that delivers a set percentage of anesthetics into the chamber. After initial equilibration of the N_2O/oxygen/isoflurane or air/isoflurane atmosphere inside the chamber, the composition of the chamber gas was analyzed by mass spectrometry for N_2O or nitrogen, isoflurane, carbon dioxide, and oxygen concentration. All experiments were approved by the Animal Use and Care Committee of the University of Virginia Health System and were conducted in accordance with the USA Public Health Service's Policy on Human Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Arterial blood gas analysis

To determine adequacy of ventilation and oxygenation, arterial blood was sampled after 2, 4 and 6 h of anesthesia (as indicated in Table 1) by obtaining a single sample (100 μl) from the left cardiac ventricle using a 32 gauge hypodermic needle (Jevtovic-Todorovic et al., 2003). Bicarbonate concentration (millimoles per liter), oxygen saturation (%), pH, $paCO_2$ (partial pressure of carbon dioxide in mm Hg), and paO_2 (partial pressure of oxygen in mm Hg) were measured immediately after blood collection, using a Nova Biomedical blood gas apparatus (Waltham, MA, USA). Control samples were obtained from air/DMSO-treated pups. The comparisons among the groups were performed using one-way ANOVA followed by Newman-Keuls test.

Histopathological studies

All pups were deeply anesthetized and perfused with aldehyde fixative for histopathology studies of the brain. We have found that activated caspase-3 immunochemical staining (ICC) is an excellent method for marking neurons that are in an early stage of apoptosis, and DeOlmos silver staining is very useful for mapping patterns of cell death in the developing brain (Jevtovic-Todorovic et al., 2003). Two additional ICC methods were used for the studies of the apoptotic pathways: caspase-9, an excellent method for studying the activation of the intrinsic apoptotic pathway (Hengartner, 2000; Felderhoff-Mueser et al., 2002), and caspase-8, an excellent method for studying the activation of the extrinsic apoptotic pathway (Chinnaiyan et al., 1995; Felderhoff-Mueser et al., 2002). Pups used for studying caspase-3, -8, and -9 activation or for silver staining were perfused with a mixture of paraformaldehyde (4%) in cacodylate buffer, pH 7.4, either 2 h (caspases) or 18 h (silver) after cessation of anesthesia.

For activated caspase -3, -8, and -9 ICC, 50-μm-thick vibrotome sections were washed in 0.01 M phosphate-buffered saline (PBS), quenched for 10 min in a solution of methanol containing 3% hydrogen peroxide, then incubated for 1 h in blocking solution (2% BSA/0.2% milk/0.1% Triton X-100 in PBS; Sigma-Aldrich Chemical Co.), followed by incubation overnight in caspase specific antibodies [e.g. rabbit anti-active caspase-3, caspase-8 and caspase-9 anti-serum (D175, Cell Signaling Technology, Beverly, MA, USA)] diluted 1:1000 for caspase-3 and caspase-8 and 1:2000 for caspase-9 in blocking solution. Following incubation with D175 primary antibody, the sections were incubated for 1 h in secondary antibody (goat anti-rabbit 1:200 in blocking solution), then reacted in the dark with ABC reagents (standard Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA), pre-incubated for 10 min in a filtered mixture containing 6 ml of 0.1 M Tris buffer, 2 mg 3,3′-diaminobenzidine (DAB) and 400 mg imidazole, and then for 15 min in 6 ml of the same DAB/imidazole/Tris mixture containing 3 μl H_2O_2. For silver staining, the fixed brains were cut by vibrotome into 50 μm sections and stained by the DeOlmos cupric silver method, as described previously (Jevtovic-Todorovic et al., 2003; DeOlmos and Ingram, 1971).

Quantitative histology

To determine the degree of neurodegeneration in a given brain region, we used the optical disector and fractionator method (West, 1999). A counting frame (0.05×0.05 mm, disector height, 0.05 mm) and a high numerical aperture objective lens were analyzed to visualize neurons. Unbiased sampling of each brain region was performed by randomly selecting 10–12 viewing fields over which the counting frame was positioned for counting at different focal levels (Stereo Investigator System, MBF, VT, USA). The numerical density of degenerating neurons in any given region was determined by counting argyrophilic profiles in 50-μm-thick sections stained by the DeOlmos silver method and ex-