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General Anesthesia Causes Long-Lasting Disturbances in the Ultrastructural Properties of Developing Synapses in Young Rats

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Abstract

Common general anesthetics administered to young rats at the peak of brain development cause widespread apoptotic neurodegeneration in their immature brain. Behavioral studies have shown that this leads to learning and memory deficiencies later in life. The subiculum, a part of the hippocampus proper and Papez's circuit, is involved in cognitive development and is vulnerable to anesthesia-induced developmental neurodegeneration. This degeneration is manifested by acute substantial neuroapoptotic damage and permanent neuronal loss in later stages of synaptogenesis. Since synapse formation is a critical component of brain development, we examined the effects of highly neurotoxic anesthesia combination (isoflurane, nitrous oxide, and midazolam) on ultrastructural development of synapses in the rat subiculum. We found that this anesthesia, when administered at the peak of synaptogenesis, causes long-lasting injury to the subicular neuropil. This is manifested as neuropil scarcity and disarray, morphological changes indicative of mitochondria degeneration, a decrease in the number of neuronal profiles with multiple synaptic boutons and significant decreases in synapse volumetric densities. We believe that observed morphological disturbances of developing synapses may, at least in part, contribute to the learning and memory deficits that occur later in life after exposure of the immature brain to general anesthesia.

Keywords

Isoflurane; Nitrous oxide; Midazolam; Synaptogenesis; Subiculum; Neurotoxicity

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Introduction

The exposure of very young children, including premature babies, to general anesthesia is becoming common. The frequency of surgery on these patients has increased, as has the length of their stays in intensive care units. Heroic attempts to save premature and extremely ill infants have resulted in their prolonged deep sedation and repeated anesthesia during an extremely delicate period of human brain development.

In the absence of evidence indicating otherwise, these practices have generally been considered safe. However, our work and that of others has now shown that general anesthetics are damaging to developing mammalian brains. It is becoming widely accepted that common general anesthetics known to potentiate inhibitory transmission through $GABA_A$ receptors (e.g., intravenous anesthetics such as benzodiazepines, barbiturates, and propofol, as well as inhalational anesthetics such as isoflurane) (Franks 2008) and/or to decrease excitatory transmission through *N*-methyl-D-aspartate (NMDA) glutamate receptors (e.g., nitrous oxide and ketamine) (Jevtovic-Todorovic et al. 1998; Lodge and Anis 1982) cause widespread apoptotic neurodegeneration when used at the peak of brain growth. This has been shown in various mammalian species, including rats, mice, guinea pigs, and nonhuman primates (Jevtovic-Todorovic et al. 2003; Ikonomidou et al. 1999, 2000; Rizzi et al. 2008; Slikker et al. 2007; Young et al. 2005). Based on behavioral studies of rats, it appears that exposure to general anesthesia at the peak of synaptogenesis causes learning and memory deficiencies later in life (Jevtovic-Todorovic et al. 2003).

The subiculum, a part of the hippocampus proper and Papez's circuit, is intertwined with the hippocampal CA1 region, anterior thalamic nuclei, and both entorhinal and cingulate cortices. Accordingly, the involvement of subiculum in cognitive processing has long been recognized based on selective lesioning studies which have shown that rats with damaged subiculum demonstrate significant impairment of spatial learning/memory and the integration of geometric information (Naber et al. 2000; Laxmi et al. 1999; Oswald and Good 2000; Devi et al. 2003). The conclusion that the subiculum is vulnerable to anesthesia-induced developmental neuroapoptosis is based not only on substantial acute neuroapoptotic damage after the administration of anesthesia (Jevtovic-Todorovic et al. 2003; Rizzi et al. 2008), but also on permanent neuronal loss in later stages of synaptogenesis (Rizzi et al. 2008; Nikizad et al. 2007).

Since anesthesia-induced neurotoxicity in the developing brain results in long-term cognitive impairment and the subiculum appears to be one of the vulnerable brain regions, we examined whether exposure to highly neurotoxic anesthesia combination containing isoflurane, nitrous oxide, and midazolam, at the peak of brain development, can cause long-lasting ultrastructural changes in the developing synapses of the rat subiculum. Synapse formation is the hallmark of synaptogenesis, a complex phenomenon that encompasses several other important elements (e.g., neuronal migration, differentiation, and maturation, as well as glial proliferation, establishment of neuronal-glial interactions, maturation/stabilization of synapses and activity dependent pruning of inappropriate synaptic contacts), which are intertwined and precisely timed (Waites et al. 2005; Munno and Syed 2003).

Materials and Methods

Animals

We used Sprague-Dawley rat pups at the postnatal day (PND) 7 for all experiments, since this is when they are most vulnerable to anesthesia-induced neuronal damage (Yon et al. 2005). Experimental rats were exposed to 6 h of anesthesia; controls were exposed to 6 h of mock anesthesia (vehicle). After the administration of anesthesia, rat pups were allowed to

recover and were reunited with their mothers. To assess their general appearance and body weight gain a large population study was first conducted ($n = 36$ controls, $n = 36$ experimentals, total of 13 litters). On PND 21, $n = 4$ control and 4 experimental pups from two litters were randomly selected for ultrastructural analysis of the subiculum. While the body weight analysis required higher number of animals to address the innate individual variability of daily weight gain, the number of animals used for complex and time-consuming ultrastructural histological studies was sufficiently powered to make proper conclusions (Dudanova et al. 2007; Kovalenko et al. 2006; Kurt et al. 2004; Uranova et al. 1991). All experiments were approved by the Animal Use and Care Committee of the University of Virginia Health System and were done in accordance with the Public Health Service's Policy on Human Care and Use of Laboratory Animals. Efforts were made to minimize the number of animals used.

Anesthesia

N_2O and oxygen were delivered using a calibrated flowmeter. Isoflurane was administered using an agent-specific vaporizer that delivers a set percentage of anesthetic into the anesthesia chamber in which rat pups were placed. Midazolam (Sigma-Aldrich Chemical, St. Louis, MO) was dissolved in 0.1% dimethyl sulfoxide (DMSO) just before administration. For control animals, 0.1% DMSO was used alone. To administer a specific concentration of N_2O/oxygen and isoflurane in a highly controlled environment, an anesthesia chamber was used (Yon et al. 2005, 2006; Lu et al. 2006). Rats were kept normothermic throughout the experiment, as previously described (Jevtovic-Todorovic et al. 2000). For control experiments, air was substituted for the gas mixture. After initial equilibration of the N_2O/oxygen/isoflurane or air atmosphere inside the chamber, the composition of the chamber gas was analyzed by mass spectrometry to establish the concentrations of N_2O or nitrogen, isoflurane, carbon dioxide, and oxygen. Our standard general anesthesia protocol consisting of a single injection of midazolam (9 mg/kg, i.p.) followed by 6 h of nitrous oxide (75%) and isoflurane (0.75%) administered to PND 7 rat pups was chosen since this protocol has been shown in several studies to cause severe apoptotic damage to the developing neurons (Jevtovic-Todorovic et al. 2003; Yon et al. 2005, 2006; Nikizad et al. 2007; Lu et al. 2006).

Histopathological Studies

On PND 21, each pup was deeply anesthetized with nembutal (65 mg/kg, i.p.). After cannulating the left ventricle, we clamped the descending aorta and did an initial flush with Tyrodes solution (30–40 ml). This was followed by 10 min of continuous perfusion with freshly prepared paraformaldehyde (4%) and glutaraldehyde (0.5%) (Jevtovic-Todorovic et al. 2003; Yon et al. 2005, 2006; Lu et al. 2006). We removed the brains and stored them in the same fixative overnight. Both control and experimental pups were perfused by an experienced experimenter on the same day, using the same solution to assure uniform tissue fixation. Any brains considered to have been inadequately perfused were not processed for EM analysis. Fixed brains were coronally sectioned (50–75 μm thick) with a DTK-1000 microslicer (Ted Pella, Tools for Science and Industry, Redding CA). The subiculum was localized as described in anatomical maps (Paxinos and Watson 1944), fixed in 1% osmium tetroxide (EM Sciences, Hatfield, PA), stained with 4% uranyl acetate (EM Sciences, Hatfield, PA) and embedded in aclar sheets using epon-araldite resins. The subiculum was then dissected from the aclar sheets and re-embedded in beam capsules. To prepare capsules for microtome cutting (Sorvall MT-2 microtome, Ivan Sorvall, Norwalk, CT) the tips were manually trimmed so that ultrathin slices (silver interference color, 600–900 Å) could be cut using a diamond knife (Diatome, Hatfield, PA). Ultrathin sections were placed on grids and examined using a 1230 JEOL TEM electron microscope, with a 16 M-pixel digital camera (SIA-12C digital cameras, Scientific Instruments and Applications, Duluth, GA). We took

12 random, nonoverlapping electron micrographs (12,000× magnification) of each subicular layer (pyramidal, polymorphic, and molecular) for each animal and derived the average number of synapses/layer for each individual. The means for each animal were then compiled ($n = 4$ control and $n = 4$ experimental pups) to calculate the mean and standard error for each subicular layer in each group (control and experimental). Since our focus was on synapses, our electron micrographs mainly depicted neuropil; large pyramidal neurons, phagocytes, and blood vessels were avoided. The investigator taking electron micrographs was blinded to the experimental conditions.

Quantitative Histology

To examine morphological properties and calculate synaptic densities, synapses were identified and counted when all of the following criteria were met: the presence of a postsynaptic density; the presence of more than one synaptic vesicle closely apposed to the presynaptic membrane; and the presence of a synaptic cleft delineated by parallel pre- and postsynaptic membranes. The number of synapses per unit volume was expressed as volumetric density (# of synapses/μm^3), using the following ratios (Erisir and Harris 2003):

1. Volumetric density = Raw density/mean length of synapses
2. Raw density = Crude number of synapses/sum of areas examined

Further classification of synapses involved identification of the asymmetric synapses, generally considered to be excitatory, and symmetric synapses, generally considered to be inhibitory. Asymmetric and symmetric synapses were defined as specified by Crain et al. (1973). Namely, symmetric synapses were identified as having pre- and post-synaptic densities of equal width, whereas asymmetric synapses were identified as having a postsynaptic density 2–3 times as thick as the presynaptic density.

When the criteria were not clearly met, the synapse was assigned to the “undetermined” category. To study different types of synaptic connections, we classified synapses into three categories: (1) Axo-spinous synapses, or synapses on the dendritic spine, were identified as those on profiles that do not contain mitochondria or microtubules, although a spine apparatus occasionally is present; (2) Axo-dendritic synapses, or synapses on dendritic shafts, were identified by the presence of microtubules or mitochondria; (3) Others, or ones that did not conform to the preceding criteria (about 3% of all synapses).

Morphometric Analyses of Synapses

All measurements were done using Image-Pro.Plus 6.1 computer software (MediaCybernetics, Bethesda, MD). The length of synapses was measured along parallel-aligned plasma membranes. If a synapse was perforated, its synaptic length included the length of the perforation. A terminal bouton in contact with two postsynaptic profiles was treated as two different synapses. The thickness of postsynaptic densities (PSDs) was calculated as the mean of two to three (depending on the length of the synapse) measurements perpendicular to the postsynaptic membrane. The number of presynaptic vesicles was the number of all vesicles that could be counted in the presynaptic terminal area (Erisir and Harris 2003).

Counting was done by an experienced histopathologist who was unaware of the treatment condition.

Statistical Analysis

Comparisons among the groups in histological studies were made using Student’s *t*-tests. To conduct the analysis of daily weight gain we used two-way ANOVA (PND and

experimental condition) followed by Bonferroni test. Significance was set at $P < 0.05$ (Graph Pad Prizm 5, Graph Pad Software Inc, La Jolla, CA).

Results

Since proper nutritional status is critically important during the development of synapses, we closely followed rat pups' general appearance and daily weight by conducting a large population study. There was no significant difference in weight gain between control and experimental animals during the first 21 days of their postnatal life (Fig. 1). In fact, based on their general appearance, the 36 control and 36 experimental animals (from 13 litters) could not be distinguished. The time points when the animals were treated (PND 7) and euthanized (PND 21) are indicated in Fig. 1.

On ultrastructural examination of the three layers of the subiculum, we found striking differences in the overall appearance of the neuropil in control and anesthesia-treated animals at PND 21 (14 days after treatment). In control rats, the neuropil was a continuum with abundant, tightly packed neuro-glial profiles. The neuropil was also rich in synaptic contacts (Fig. 2A, a, black arrows). In the experimental neuropil, we found numerous scant and empty-looking areas with multiple "blank" spaces containing only cellular debris, broken-looking profiles, and scarce synapses (Fig. 2A, b, c).

In addition to the changes in the appearance of the neuropil, we observed ultrastructural changes indicative of degeneration in mitochondria in the experimental subiculum (Fig. 2B). Some appeared swollen, with balloon-like cristae, but had normal-looking inner and outer membranes (early stage, Fig. 2B, e). There was also an abundance of dark, condensed, and shrunken mitochondrial profiles having no clear outline between the inner and the outer membrane (late stage, Fig. 2B, f).

To assess whether scarcity of neuropil resulted in a decrease in synapse density in anesthesia-treated rat pups, we quantified the overall number of synapses per μm^3 in each subicular layer and expressed this number as volumetric density (see "Materials and Methods") (Erisir and Harris 2003). We found an average of 30–40% lower synapse volumetric density in each subicular layer of anesthesia-treated rats than in control rats ($n =$ 4 control and 4 experimental pups from two different litters) (Fig. 3). This lowered synapse density attained statistical significance.

An interesting feature of the experimental neuropil in PND 21 animals was the scarcity of multiple synaptic boutons, defined as synapses formed between an individual axon and more than one neuronal profile (Sorensen et al. 2003; Yankova et al. 2001; Briones et al. 2004; Wooley et al. 1996). It was more common to encounter multiple synaptic boutons (showing 2–3 synapses in average) in control animals (Fig. 4A), whereas even normal-looking presynaptic terminals in experimental rats made only one or no synaptic contacts (Fig. 4B). In addition, our ultra-structural analysis of the experimental subiculum showed that some synapses that appeared morphologically intact were found in degenerating neuronal profiles (Fig. 5).

To examine whether the observed difference in volumetric densities is due to preferential loss of excitatory or inhibitory synapses, we quantified the overall number of inhibitory and excitatory synapses based on the established morphological criteria (see "Materials and Methods") and compared relative changes between control and experimental animals. When a clear distinction could not be made, the synapses were defined as "undetermined." As shown in Fig. 6, we found no differences in relative ratios among the excitatory, inhibitory, and undetermined synapses in the subicular layers. Thus, although the overall synapse density was significantly decreased in experimental animals, the ratios among the excitatory,

inhibitory, and undetermined synapses spared by anesthesia remained the same as the ratios in controls (approximately 75, 5, and 20%, respectively). To investigate the possibility that anesthesia could be preferentially more damaging to certain types of synaptic contacts, we examined the relative ratios among axo-spinous, axo-dendritic, and other types of synapses in experimental animals, but found no difference compared to the ratios in controls. On average 70–80%, 15–20%, and 5–10% of synapses were axo-spinous, axo-dendritic, and others, respectively, in both control and experimental rats.

To determine whether anesthesia causes morphological changes in subicular synapses, we measured synaptic lengths, the thickness of postsynaptic densities, the number of presynaptic vesicles and the areas of presynaptic terminals in pyramidal, molecular, and polymorphic layers. We found no significant differences in these characteristics of synapses in experimental and control rats (data not shown).

Discussion

Our results demonstrate that exposure of immature rats to general anesthesia at the peak of synaptogenesis causes severe and long-lasting ultrastructural abnormalities of young neurons in the developing subiculum. In addition to the scarcity of neuropil and degeneration of mitochondria, we observed significant decreases in synapse volumetric densities and the paucity of multiple synaptic boutons in subicular neurons in the pyramidal, polymorphic, and molecular layers 2 weeks after the administration of anesthesia. The subiculum, strategically positioned between the hippocampus and the entorhinal cortex, is considered a part of the hippocampal formation (O'Mara et al. 2001). It is functionally and anatomically intertwined with the CA1 region of the hippocampus. As such, it mediates a myriad of hippocampal-cortical and hippocampal-subcortical interactions, especially with anterior thalamic nuclei (McNaughton 2006). Previously, we have shown that the immature subiculum in rats and guinea pigs is vulnerable to anesthesia-induced apoptotic acute neurodegeneration (Jevtovic-Todorovic et al. 2003; Rizzi et al. 2008) and that this neuronal damage results in permanent neuronal deletion in the final stages of synaptogenesis (Rizzi et al. 2008; Nikizad et al. 2007). Here we show that anesthesia-induced neuronal damage goes beyond acute neuronal destruction, leaving many neurons dysfunctional and with ultrastructural abnormalities that persist many days after anesthesia exposure, suggesting that a single exposure to anesthesia has far-reaching effects on synaptic organization in the subiculum.

Specific lesions of the subicular complex impair cognitive function in rodents, having particularly devastating effects on long-term spatial learning and memory (Morris et al. 1990; Galani et al. 1998). Earlier, we found that the subiculum is sensitive to anesthesia-induced neurodegeneration, not only soon after its administration, but also later in adult life, as demonstrated by significant spatial learning and memory deficits (Jevtovic-Todorovic et al. 2003). Here we have shown that the same anesthesia combination causes protracted neuronal degeneration in the subiculum, leading to significant synapse scarcity. Since synaptic connections between the subiculum and components of Papez's circuit are considered to be important for proper cognitive development (McNaughton 2006; Chua et al. 2006), it is possible that the anesthesia-induced synaptic disturbances we have demonstrated may, at least in part, explain previously reported cognitive impairments in rodents exposed to general anesthesia early in life (Jevtovic-Todorovic et al. 2003).

It is of interest that those cognitive impairments appear to be positively correlated with the histo-pathological finding in the cases when the damage is protracted, leading to permanent synaptic disturbances and neuronal loss, and not when anesthesia-induced neuronal damage is transient. For example, a recent study by Loepke et al. (2009) has shown that despite the

fact that isoflurane anesthesia causes significant short-term apoptotic neurodegeneration in the immature mouse brain, it does not lead to neuronal loss and/or neurocognitive impairments later in life.

The most striking ultrastructural features of anesthesia-treated subicular tissue were the scarcity of neuro-glial profiles and the empty-looking neuropil, even 14 days after exposure to anesthesia. It would be reasonable to expect that in active young brains quick pruning and removal of cellular debris would occur soon after an insult, with reconstruction giving rise to a normal-looking neuropil. Indeed, Oo et al. (1995) have shown that in PND 7 rat pups, hypoxic-ischemic insult, which causes massive neuronal death, is followed by rapid removal of dead cells, resulting in a normal-looking neuropil within about 4 days. Similarly, localized brain lesions caused by head trauma in PND 7 rats result in removal of dead cells and normal-looking tissue at 5 days after trauma (Bittigau et al. 1999). In contrast, we found ill-looking neuropil with many neuro-glial profiles undergoing degeneration 14 days after insult. Although anesthesia-induced neuronal death has been reported to be apoptotic and, accordingly, may take time to evolve (Yon et al. 2005, 2006), it appears that there must be some additional explanation for what appears to be ongoing neuropil demise. We hypothesize that the significant decrease in synapse densities could affect the ability of the neurons to interconnect and form meaningful and functional circuitries. This could cause a cycle of ongoing recruitment of redundant neurons that are unable to function properly and therefore are destined to die. Further studies of anesthesia-induced effects on other aspects of synaptogenesis, including neuronal migration, differentiation, and maturation, are needed to address other possible explanations for protracted neuropil demise after a single anesthesia exposure.

An interesting feature of the experimental neuropil was the paucity of multiple synaptic boutons (MSBs) in all layers of the experimental subiculum when compared to controls. MSBs are defined as synapses formed between an individual axon and more than one neuronal profile, as opposed to single synapse boutons (SSBs), which are synaptically connected to only one postsynaptic neuronal profile (Briones et al. 2004; Sorensen et al. 2003; Yankova et al. 2001; Wooley et al. 1996). The abundance of MSBs either from the “same cell” or “different cell” contacts is believed to predict increased efficiency of synaptic transmission and/or improved synchronization of synaptically driven activity (Toni et al. 2007; Yankova et al. 2001; Briones et al. 2004), with an overall increase in structural stability and synaptic efficacy when compared to single synaptic connections. Although our study was not designed to trace multiple synapses to their neuronal profiles of origin it is reasonable to propose that anesthesia-induced scarcity of MSBs, whether of the “same” or “different” cell contacts, could potentially contribute to previously reported neurocognitive impairment (Jevtovic-Todorovic et al. 2003).

The importance of proper nutrition during synaptogenesis has been well-established. It was suggested, more than 20 years ago, that there is an association between poor nutrition during early life, alteration in cognitive development and decreased synapse densities in hippocampal formation (Ahmed et al. 1987). That suggestion has since been confirmed (Granados-Rojas et al. 2004; Lister et al. 2005). Of special interest to our study are the findings regarding the effects of prolonged (30-day) undernutrition on the developmental growth curve for ratios of synapses to neurons, which suggest a decline in the complexity of synapsing in the presence of malnutrition (Peeling and Smart 1994). To assure that short nutritional deprivation during and immediately after anesthesia (between 6 and 7 h) had no effect on the growth curve, we made sure that control animals were exposed to the same short-term undernutrition as were experimental animals. Daily weight gain, closely followed until the time of sacrifice, confirmed that rats’ nutritional development was unaffected. Thus, we conclude that the observed synaptic abnormalities are unlikely to have been caused

by the short period of undernutrition but, instead, most likely are caused by long-lasting anesthesia-induced impairment of synaptogenesis.

Selective synapse loss and neuronal loss have been reported in pathological conditions such as chronic degenerative diseases (Day et al. 2006; Lacor et al. 2004) and ischemic and reperfusion injuries (Garcia et al. 1996). For example, in primates with ischemia-reperfusion injury a selective loss of inhibitory synapses has been reported to occur in cortical brain regions that were not infarcted, suggesting that certain brain injury paradigms can target a selective group of synapses and/or neurons (Giffard et al. 2008). Based on our observations, anesthesia-induced depletion of synapses does not appear to be selective for any layer of subicular neurons. Also, although the synapse loss is significant, it equally targets different types of synaptic contacts (axo-spinous, axo-dendritic, and others). We have previously reported that general anesthesia causes neuronal deletion in the subiculum of both rats and guinea pigs (Rizzi et al. 2008; Nikizad et al. 2007), causing approximately 30–40% neuronal loss as compared to age-matched controls (Nikizad et al. 2007). This suggests that nonselective synaptic loss after anesthesia exposure is likely due to an overall neuronal loss.

The effect of volatile inhalational anesthetics on synaptic development was studied many years ago (Quimby et al. 1974; Crain et al. 1973; Chang et al. 1974, 1976; Uemura et al. 1985). The focus then was on halothane, a volatile anesthetic similar to isoflurane. Also, the experimental exposure was low-level but chronic, in an attempt to simulate the exposure to low atmospheric concentrations of volatile anesthetics experienced in utero by the offspring of pregnant operating room personnel. Despite the obvious difference in the length of exposure (6 h in our study versus all of in utero life and 28 days postnatally for 8 h/day 5 days a week), the similarities in the ultrastructural neuronal damage are striking. Various authors have reported significant reductions in cortical and subicular volumetric synaptic densities, with lags in synaptic development and maturation, as well as sparse-looking neuropil (Quimby et al. 1974; Chang et al. 1976; Crain et al. 1973; Uemura et al. 1985). Although our study did not follow the ultrastructural changes beyond the main synaptogenesis period (the first 3 weeks of postnatal life), other authors have reported halothane-induced neuronal degeneration and organelle abnormalities in the cerebral cortex more than 3 months after chronic exposure (Chang et al. 1976). Interestingly, chronic exposure to halothane resulted in a significant impairment of the development of postsynaptic membrane densities (Quimby et al. 1974). We did not find that similar changes were induced by short exposure to isoflurane in combination with midazolam and nitrous oxide.

Despite the apparently normal appearance of subicular tissue at the light microscopic level (Nikizad et al. 2007), long-term impairment of normal neuronal development becomes obvious when studied at the ultrastructural level, suggesting that subtle, insidious, and ongoing neuropil demise occurs many days after anesthesia exposure. To our knowledge, this is the first report of lasting synaptic plasticity in the young rodent brain after single exposure to anesthesia early in life. Using extracellular recordings from CA1 hippocampal neurons in slices, we have previously demonstrated that the same anesthetic combination as that used in the present study (isoflurane, midazolam, and nitrous oxide) caused lasting depression of long-term potentiation (LTP), but not depression of excitatory synaptic strength (Jevtovic-Todorovic et al. 2003). This is interesting, considering that our morphometric studies of the subiculum show nonselective synapse loss. The obvious reason for potentially selective functional synaptic plasticity is not known. However, it is of interest that functioning mitochondria and their ATP production are essential for maintaining normal synaptic physiology (Li et al. 2004; Inquimbert et al. 2008; Jonas 2004). Acute application of isoflurane or midazolam potentiates the inhibitory drive by heightening inhibitory synaptic activity mediated by $GABA_A$ receptors (Franks 2008). In contrast, nitrous oxide

silences excitatory (NMDA-mediated) synaptic transmission (Mennerick et al. 1998). Thus, it is possible that anesthesia-induced degenerative changes in mitochondria and a consequent decrease in ATP production preferentially impairs highly activated inhibitory synaptic function due to an inadequate metabolic ratio of supply to demand. However, the precise mechanism for the selective homeostatic changes of neuronal function under extensive GABAergic stimulation associated with various physiological and pathological conditions remains to be examined (Mody 2005).

We conclude that anesthesia-induced impairment of synaptogenesis in the rat subiculum is marked by morphological disturbances of developing synapses. This may, at least in part, contribute to the learning and memory deficits that occur later in life after exposure of the immature brain to general anesthesia.

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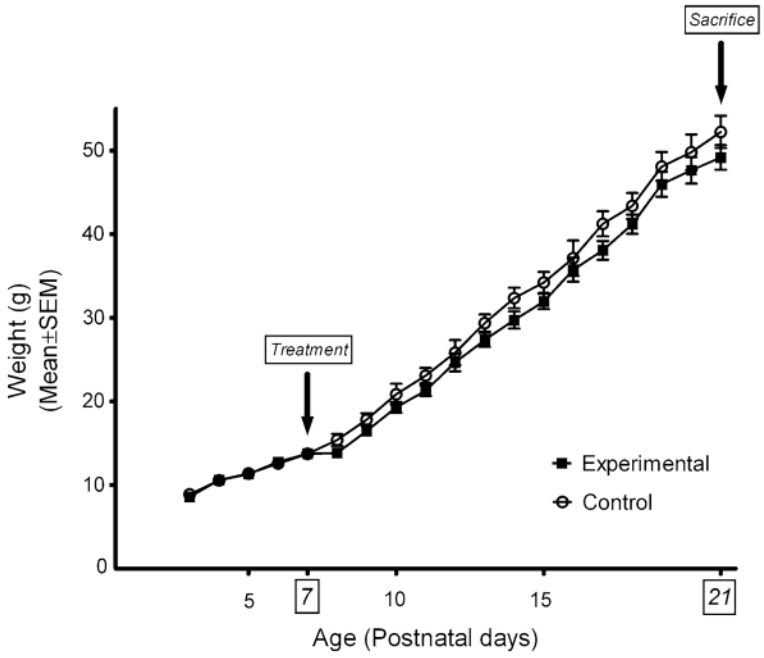

Fig. 1.

Anesthesia did not cause significant differences in weight gain between control and experimental rats during the first 21 days of postnatal life. Daily weight measurements before and after anesthesia exposure (marked with an *arrow* as Treatment) show no difference in weight gain between control and experimental rats up to 14 days after anesthesia (PND 21) (marked with an *arrow* as Sacrifice) (n = 36 control and 36 experimental animals total)

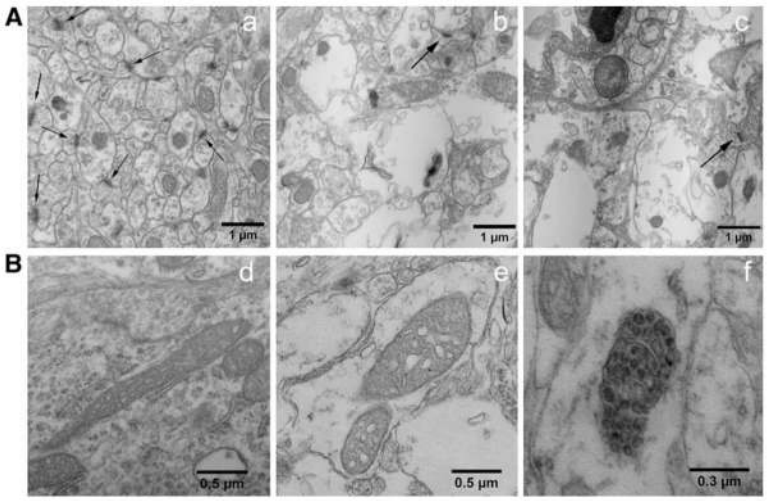

Fig. 2.

Anesthesia caused long-lasting ultrastructural disarray in the subicular neuropil (**A**, *upper panel*) and mitochondria (**B**, *lower panel*) of 21-day-old rats. **a** The pyramidal layer of a subiculum from a control rat shows the abundance of tightly packed neuro-glial profiles with numerous synaptic contacts (*arrows*). **b, c** The pyramidal layers of subiculums from two different rats from different litters show a striking scarcity of synaptic contacts (two synaptic contacts are indicated by *arrows* in **b** and **c**) and a gross loss of neuro-glial profiles. **d** Mitochondria in a control subiculum appear healthy with a clear double membrane and tight, orderly cristae. **e, f** Experimental mitochondria exhibited various stages of degeneration. In the early stage, they appeared swollen, with balloon-like cristae, but had normal-looking inner and outer membranes (**e**). In the later stage they appeared dark, condensed, and shrunken, with no clear outline between the inner and the outer membranes (**f**) (magnification × 12,000)

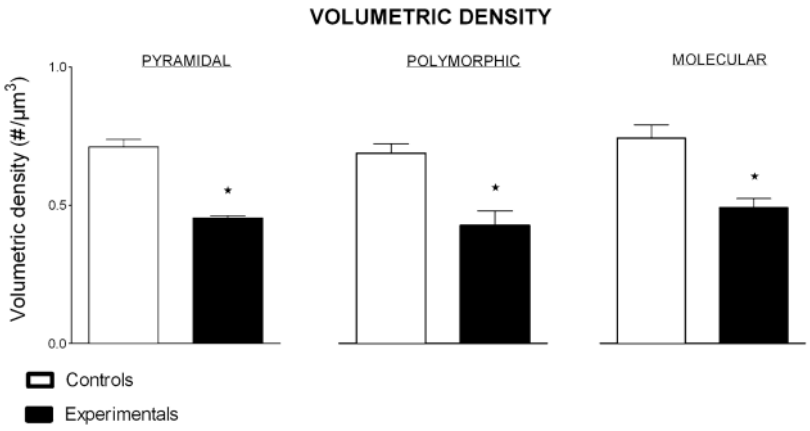

Fig. 3.

Anesthesia caused significant decrease in volumetric density in all three subicular layers of 21-day-old rats. When the overall number of synapses per μm^3 was quantified in the pyramidal, polymorphic, and molecular layers, there was significantly less synapse density in each subicular layer of anesthesia-treated animals (on average 30–40%) as compared to controls (*pyramidal layer $P < 0.001$; *polymorphic layer and molecular layers, $P < 0.01$) (n = 4 control and 4 experimental pups from two litters)

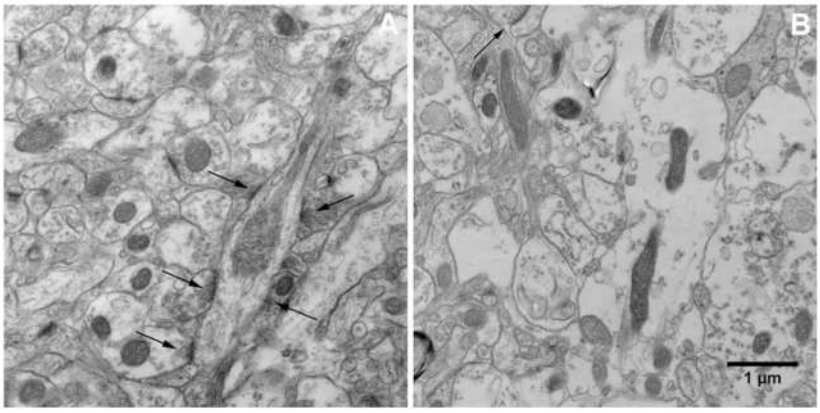

Fig. 4.

Anesthesia-treated animals had scarce multiple synaptic boutons (MSBs). **A** Shows the abundance of MSBs (arrows) on the neuronal profile of a control subiculum. In the experimental subiculum (**B**) there is, in contrast, a substantial lack of synaptic contacts (magnification × 12,000)

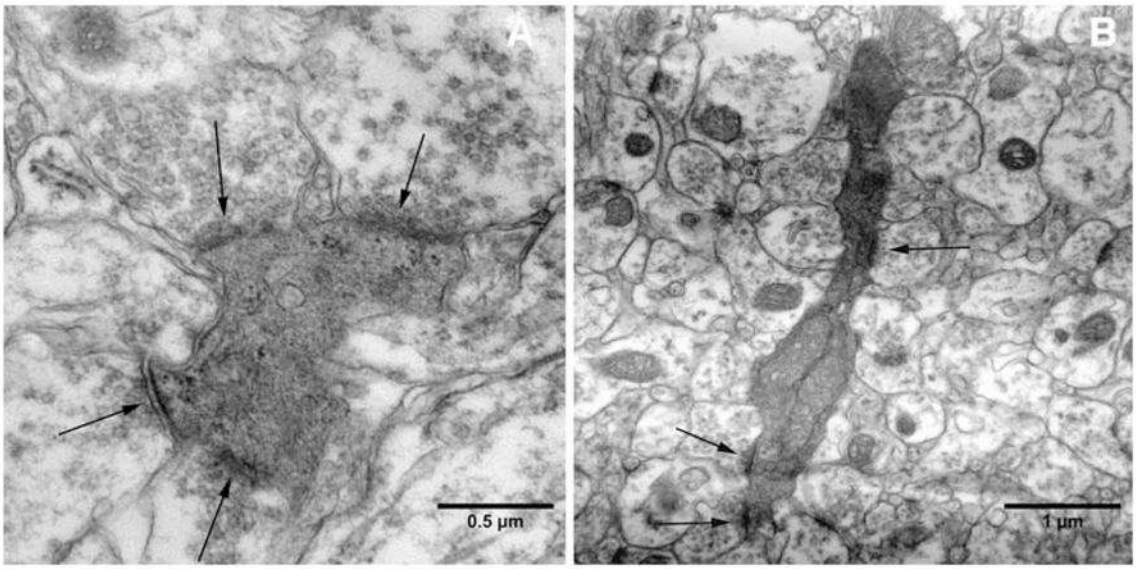

Fig. 5.

Morphologically intact-looking synapses were found on degenerating neuronal profiles in anesthesia-treated 21-day-old rats. Multiple synapses (*black arrows*, *left* and *right* panels) with numerous docked vesicles were engaged on condensed neuronal profiles that were undergoing degeneration (magnification × 12,000)

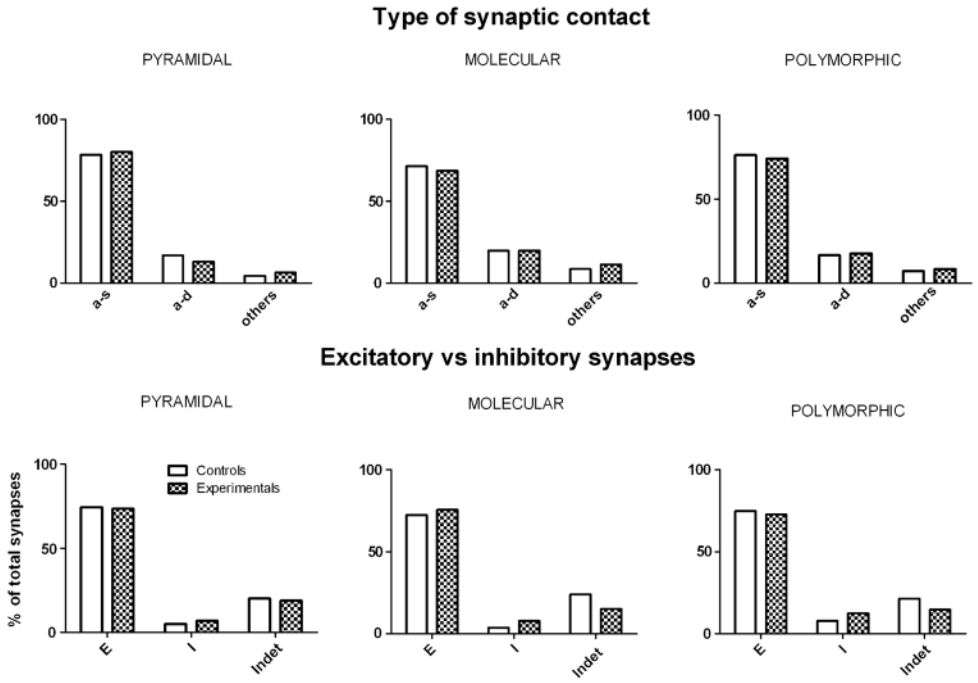

Fig. 6.

Anesthesia did not cause preferential loss of different types of synaptic contacts (*upper panel*) or preferential loss of excitatory versus inhibitory synapses (*lower panel*). There was no difference in the relative ratio of excitatory, inhibitory, and undetermined synapses in any subicular layer. The ratio among excitatory, inhibitory, and undetermined synapses spared by anesthesia remained the same as in controls; i.e., approximately 75, 5, and 20%, respectively. Similarly, there was no difference in the relative ratios among axo-spinous, axo-dendritic, and other types of synapses compared to the ratios in controls (on average 70–80%, 15–20%, and 5–10% of synapses were defined as axo-spinal, axo-dendritic, and others, respectively, in both controls and experimentals) ($n = 4$ controls and 4 experimental pups from two litters)