Mol Neurobiol
DOI 10.1007/s12035-015-9246-7

Early Exposure to General Anesthesia Disrupts Spatial Organization of Presynaptic Vesicles in Nerve Terminals of the Developing Rat Subiculum

N. Lunardi[1] · A. Oklopcic[1] · M. Prillaman[1] · A. Erisir[2] · V. Jevtovic-Todorovic[1]

Received: 27 May 2015 /

Abstract Exposure to general anesthesia (GA) during critical stages of brain development induces widespread neuronal apoptosis and causes long-lasting behavioral deficits in numerous animal species. Although several studies have focused on the morphological fate of neurons dying acutely by GA-induced developmental neuroapoptosis, the effects of an early exposure to GA on the surviving synapses remain unclear. The aim of this study is to study whether exposure to GA disrupts the fine regulation of the dynamic spatial organization and trafficking of synaptic vesicles in presynaptic terminals. We exposed postnatal day 7 (PND7) rat pups to a clinically relevant anesthetic combination of midazolam, nitrous oxide, and isoflurane and performed a detailed ultrastructural analysis of the synaptic vesicle architecture at presynaptic terminals in the subiculum of rats at PND 12. In addition to a significant decrease in the density of presynaptic vesicles, we observed a reduction of docked vesicles, as well as a reduction of vesicles located within 100 nm from the active zone, in animals 5 days after an initial exposure to GA. We also found that the synaptic vesicles of animals exposed to GA are located more distally with respect to the plasma membrane than those of sham control animals and that the distance between presynaptic vesicles is increased in GA-exposed animals compared to sham controls. We report that exposure of immature rats to GA during critical stages of brain development causes significant disruption of the strategic topography of presynaptic vesicles within the nerve terminals of the subiculum.

Keywords Developmental neurotoxicity · Hippocampus · Isoflurane · Nitrous oxide · Midazolam · Learning and memory

Introduction

Exposure to general anesthesia (GA) during critical stages of brain development induces widespread neuronal apoptosis in vulnerable brain regions of numerous animal species (i.e., mice, rats, guinea pigs, piglets, and non-human primates) [1–3]. Importantly, early exposure to GA is known to also cause long-lasting behavioral deficits, most notably the impairment of higher cognitive functions, such as learning and memory [1–3]. To date, the mechanisms underlying the long-term cognitive deficits observed after exposure to GA remain unclear and the functional effects of an early exposure to GA on synaptic transmission have not been explored in detail.

We and others [4, 5] have shown that exposure to GA causes a significant decrease in synaptic densities. Of particular interest for this study is our previously published finding that GA exposure results in about a 30 % decrease in the volumetric density of synapses in the subiculum of young rats [4], a brain region critically important in cognitive and emotional development [6–9]. In addition, we have reported lasting impairment in subicular synaptic plasticity, i.e., neuropil disarray, decrease in the number of neurons with multiple synaptic boutons and mitochondria degeneration, many days after GA exposure [4]. Moreover, a recent study showed that in neuronal cultures exposed to isoflurane, injury was not only restricted to apoptosis and neuronal death, but also included loss of dendritic spines and associated excitatory synapses in

✉ N. Lunardi
nl3f@virginia.edu

[1] Department of Anesthesiology, University of Virginia Health System, PO Box 800710, Charlottesville, VA 22908, USA

[2] Department of Psychology, University of Virginia, Charlottesville, VA, USA

Published online: 06 June 2015

the remaining neurons, suggesting GA-induced modulation of synaptic architecture and consequently excitatory neurotransmission [5].

The actin cytoskeleton in presynaptic terminals is crucial in maintaining and regulating vesicle pools, by providing a scaffold to spatially segregate vesicles, as well as a conduit to transfer vesicles between pools [10–12]. Since regulation of the equilibrium between reserve and readily releasable pools of presynaptic vesicles underlies important forms of synaptic plasticity, the disruption of the actin cytoskeleton has been shown to alter synaptic efficacy at presynaptic terminals [10–12]. Interestingly, both isoflurane and propofol, two commonly used general anesthetics, have been shown to cause neurotoxicity by inducing actin depolymerization through a BDNF-p75NTR-RhoA cascade [13, 14]. RhoA is a small GTPase that is activated by p75NTR signaling; RhoA activation leads to actin cytoskeleton depolymerization and apoptosis through its effector Rho-associated kinase (ROCK) [14, 15].

In view of the evidence that general anesthetics can disrupt actin organization at the presynaptic terminals and that the actin cytoskeleton is critical for synaptic vesicle organization at presynaptic boutons, we asked whether exposure to GA could interfere with the fine regulation of the dynamic spatial organization and trafficking of synaptic vesicles in presynaptic terminals. To address this question, we exposed postnatal day 7 (PND7) rat pups to GA and performed a detailed ultrastructural analysis of the synaptic vesicle architecture at presynaptic terminals of GA-treated and sham control animals.

Materials and Methods

Animals and Anesthesia

We used Sprague-Dawley rat pups at postnatal day 7 (PND7) for all experiments, since this approximates the brain growth spurt in rats [1, 4]. Experimental rats were exposed to 6 h of general anesthesia; sham controls were exposed to 6 h of mock anesthesia. Anesthesia consisted of a clinically relevant combination of nitrous oxide (70 %), isoflurane (0.75 %), and midazolam [1, 4, 16–19]. During the administration of anesthesia, experimental animals were kept inside an anesthesia chamber, while sham control animals were kept in a separate chamber away from the mother. After the administration of anesthesia, rat pups were allowed to recover and were reunited with their mother. To administer a constant concentration of nitrous oxide, isoflurane and oxygen in a highly controlled environment, an anesthesia chamber was used, as previously described [1, 4, 16–19]. Briefly, nitrous oxide and oxygen were delivered using a calibrated flowmeter. Isoflurane was administered using an agent-specific vaporizer, delivering a set percentage of anesthetics in the anesthesia chamber. Sham control animals received vehicle (0.1 % dimethyl sulfoxide, DMSO), injected i.p., while experimental animals received i.p. midazolam 9 mg/kg (Sigma-Aldrich Chemical, St. Louis, MO), dissolved in DMSO immediately before administration. For control experiments, air was substituted for the gas mixture. For experimental animals, after initial equilibration of the nitrous oxide/isoflurane/oxygen mixture, the composition of the anesthesia chamber gases was kept constant and continuously analyzed using an infrared analyzer (Datex Ohmeda, Madison, WI). Care was taken to keep ambient temperatures constant and equal between sham control and GA-exposed animals.

To examine presynaptic nerve terminals we chose to study the developing subiculi in PND 12 rat pups to allow several days for neuropil to clear from apoptotic debris caused by acute GA exposure (at PND7). A total of four control and four experimental pups from three different litters were randomly selected for ultrastructural analysis of the presynaptic terminals. The number of animals used for time-consuming ultrastructural histological studies was sufficiently powered to make proper conclusions [20–23]. 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. Every effort was made to minimize the number of animals used.

Histopathological Studies

On PND12, pups were deeply anesthetized with isoflurane, as described previously [1, 4, 16–19]. Briefly, cannulation of the left ventricle was achieved with a beating heart and an initial flush with 100 ml of Tyrode's solution was administered. This was followed by 10 min of a continuous perfusion with freshly prepared paraformaldehyde 4 % and glutaraldehyde 2 %, dissolved in 0.1 M phosphate buffer at pH 7.4. Brains were removed from the skull and stored in fixative. All animals were perfused by an experienced experimenter on the same day and using the same solutions, in order to ensure uniform tissue fixation. Any brains deemed inadequately perfused were excluded from the analysis. Within 3 days from the initial perfusion, brains were sliced in 75-μm coronal sections with a DTK-1000 microslicer (Ted Pella, Tools for Science and Industry, Redding, CA). Our morphological analysis focused on the subiculum because, as part of the hippocampus proper, it is highly vulnerable to GA-induced developmental neurodegeneration and is pivotal in higher cognitive development, particularly for short-term and spatial memory [6–9].

The subiculum was localized as described in anatomical maps [24], fixed in 1 % osmium tetroxide, stained with 4 % uranyl acetate, dehydrated sequentially in ethanol, and flat embedded within an EPON resin in Aclar sheets at 60 °C for 48 h, as described previously [4, 25]. The subiculum was

then removed from the Aclar sheets, encapsulated in beems, and placed in an oven at 60 °C for two additional days. Once polymerized, the tips of the Beem capsules were trimmed manually to expose the area of interest and to allow for ultrathin sectioning (silver interference color, 600–900 Å) with a diamond knife (Diatome, Hatfield, PA) [4, 25]. Sections were placed in grids and post-stained with uranyl acetate and lead to facilitate the detailed morphological analysis of synaptic vesicles at presynaptic terminals. Grids were analyzed with a 1030 JEOL transmission electron microscope, equipped with a 16-megapixel digital camera (SIA-12C digital cameras, Scientific Instruments and Applications, Duluth, GA). All micrographs were taken at a magnification of ×20,000 (2×2 binning). Ten to 25 synapses were analyzed per each animal for a total of 59 synapses analyzed for sham controls and 55 analyzed for GA-exposed animals (2088 synaptic vesicles for sham controls and 1629 for experimental animals, n=4 sham control and 4 GA-exposed animals).

Morphometric Analysis of Presynaptic Terminals

All measurements were obtained using Image-Pro Plus 7.0 (Media Cybernetics, Bethesda, MD). As previously described [4], synapses were identified when all of the following criteria were met: the presence of more than one synaptic vesicle closely apposed to the presynaptic membrane; the presence of a synaptic cleft delineated by parallel pre- and postsynaptic membranes; and the presence of a postsynaptic density. Only excitatory synapses were analyzed in this study, since they represent approximately 85–90 % of all hippocampal synapses. They were identified morphologically as asymmetric synapses, i.e., having a postsynaptic density two to three times as thick as the presynaptic density, according to the characterization by Crain et al. [26]. The length of synapses was measured as the length of the postsynaptic density. If a synapse was perforated, its synaptic length included the length of the perforation. The density of synaptic vesicles at the presynaptic terminals was calculated by dividing the number of all vesicles that could be counted in the presynaptic terminal with the area of the presynaptic terminal. For vesicle density analysis, the area in the presynaptic terminal occupied by mitochondria was excluded from the cross-sectional area of the terminal [27]. The distance of a synaptic vesicle from the active zone was measured as the length of a perpendicular line traced between the center of the synaptic vesicle and a line parallel to the presynaptic membrane [22, 23]. A presynaptic vesicle was counted as docked when making contact with the presynaptic plasma membrane. The number of docked vesicles was normalized by the length of the synapse, measured as the length of the postsynaptic density. The size of a synaptic vesicle was measured as the longest diameter of the synaptic vesicle. To obtain the intervesicle distance, the X-Y coordinates of each vesicle within each synaptic bouton were determined, and the intervesicle distance was extracted from these coordinates using a Matlab algorithm (Matlab software, Mathworks), designed by James Hounshell (Neuroscience Graduate Program, University of Virginia, Charlottesville, VA). The distance between each possible pair of synaptic vesicles within each terminal was determined, and the shortest distance obtained for each vesicle pair was selected [27]. The shortest distances were then averaged per each synaptic terminal. Ultrastructural analysis was done by two experienced histopathologists who were unaware of the treatment conditions.

Statistical Analysis

Comparisons among sham control and GA-treated groups were made using Student's t test (unpaired, two-tailed, significance set at $p<0.05$). GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA) was used for statistical analysis. All data points are presented as mean±SEM.

Results

Early Exposure to GA Decreases the Density of Synaptic Vesicles in Presynaptic Terminals

For each terminal, we calculated vesicle density by counting the number of vesicles and dividing it by the cross-sectional area of each presynaptic terminal, excluding mitochondrial areas. All densities from one animal were then averaged together. We analyzed 55 and 59 presynaptic terminals in GA-treated and sham control animals, respectively.

As shown in Fig. 1a, we found a significant decrease in the density of presynaptic vesicles in animals exposed to GA compared to sham controls (*, $p<0.05$, n=4 sham control and 4 GA-treated animals). In GA-exposed animals the average number of presynaptic vesicles was 71.4±2.6 per μm^2 and in sham control animals was 98.6±6.9 per μm^2. Although the decrease in presynaptic vesicle densities could be explained by a reduction in the number of vesicles at the presynaptic terminals of GA-exposed animals, it could also reflect a change in the terminal area (i.e., an increase in the area of presynaptic terminals of GA-treated rats perhaps due to substantial swelling).

To address the question of terminal areas more thoroughly, we compared sham control and GA-exposed animals with respect to the area of their presynaptic terminals. As shown in Fig. 1b, although there was a trend towards bigger presynaptic terminal areas in GA-treated animals compared to sham controls, this trend did not reach statistical significance (p= 0.118, n=4 sham control, and 4 GA-exposed animals). The average area of subicular terminals was 0.56±0.06 μm^2 in

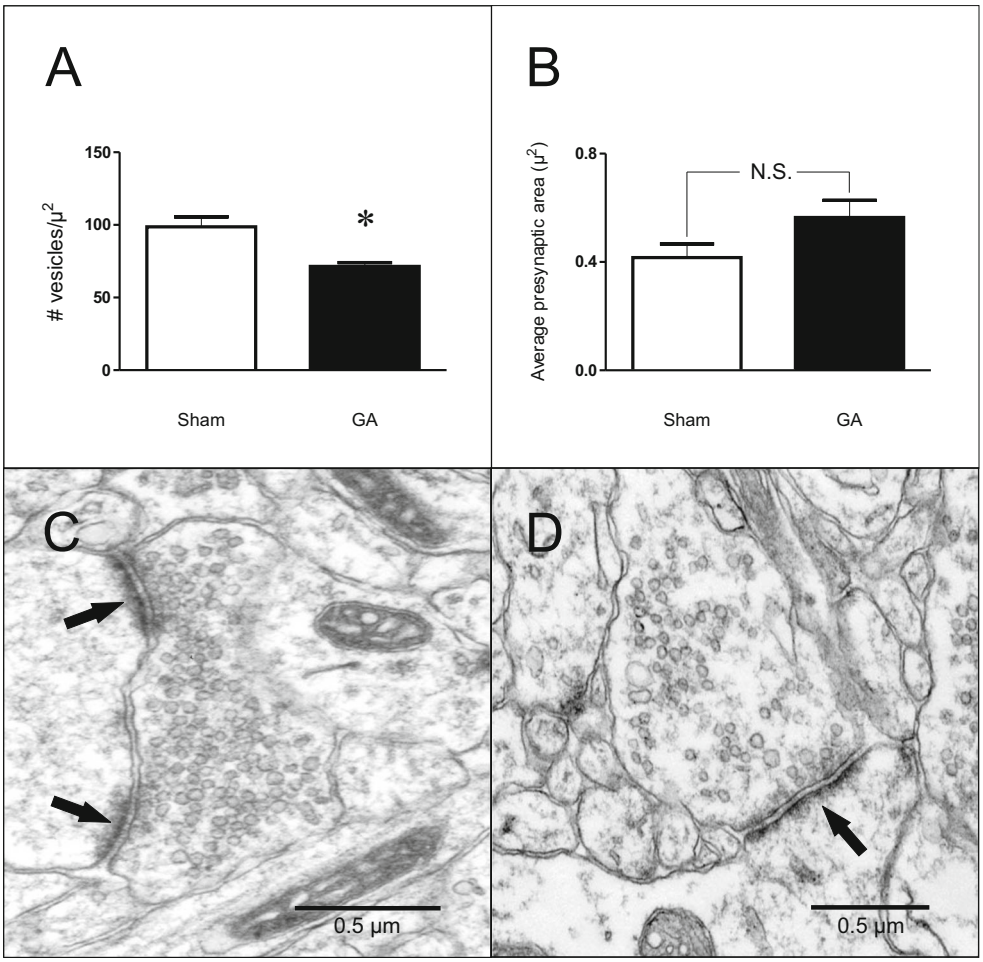

Fig. 1 Early exposure to GA decreases the density of synaptic vesicles in presynaptic terminals. **a** The number of presynaptic vesicles, normalized by the area of the presynaptic terminal, is decreased by approximately 30 % in animals exposed to GA compared to sham controls ($p<0.05$, $n=4$ sham control and 4 experimental animals from 3 litters). **b** There is no difference in the area of presynaptic terminals in anesthesia-exposed animals compared to sham controls ($p=0.12$, $n=4$ sham control and 4 experimental animals). **c**, **d** Representative electron micrographs from a sham control and a GA-treated animal, respectively. *Arrows* indicate excitatory synaptic contacts. *Calibration bars* 0.5 μm. Note that while the area of the presynaptic terminals is approximately the same, the number of presynaptic vesicles is significantly lower in the GA-exposed animal than in the sham control

GA-exposed animals and 0.41±0.05 μm^2 in sham control animals.

We conclude that the observed decrease in the density of presynaptic vesicles in GA-treated animals compared to sham controls is mainly due to a decrease in the number of vesicles in the presynaptic terminals, rather than an increase in the area of the presynaptic terminals themselves. The representative micrographs of sham control (C) and GA-treated (D) animals show that while the area of the presynaptic terminals is approximately the same, the number of presynaptic vesicles is significantly higher in the GA-exposed animal than in the sham control.

Exposure to GA Disrupts the Spatial Organization of Synaptic Vesicles in Presynaptic Terminals

To determine whether anesthesia causes changes in the spatial organization and trafficking of presynaptic vesicles, we measured the distance between each synaptic vesicle and the plasma membrane (PM). As shown in Fig. 2, there is a differential distribution of vesicles within 600 nm from PM, i.e., there were fewer vesicles in GA-treated animals in the area closest

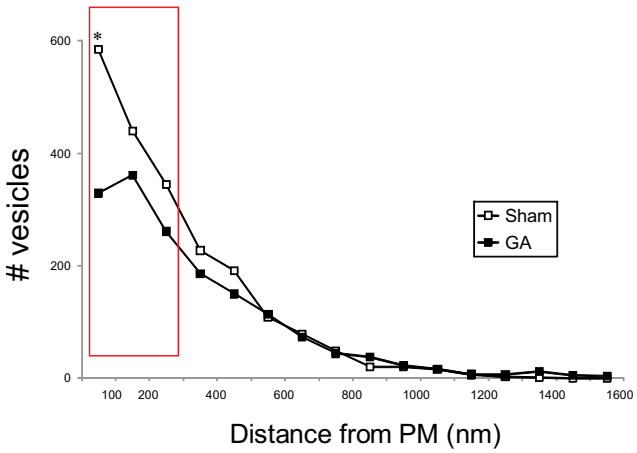

Fig. 2 Exposure to GA causes differential distribution of synaptic vesicles in presynaptic terminals. When the distribution of vesicles was analyzed in 100 nm segmental increments from the plasma membrane, we found differential distribution of vesicles within 600 nm from PM, i.e., there were fewer vesicles in GA-treated animals in the area closest to the PM. In particular, the number of vesicles located within 100 nm of the active zone was found to be significantly lower compared to sham controls (as highlighted by a red rectangle) (*, $p<0.05$, $n=4$ sham control and 4 experimental animals)

to the PM. In particular, the number of vesicles located within 100 nm of the active zone was found to be significantly lower compared to sham controls (as highlighted by a red rectangle) (*, $p<0.05$, $n=4$ sham control and 4 experimental animals).

Once we determined that a 100-nm zone is most significantly affected by an early GA exposure, we considered this area most important for our spatial analysis. As shown in Fig. 3a, we found that the average number of vesicles in this zone was 82.2±9.8 in GA-exposed animals, compared to 146.3±14.4 in sham controls. This difference was statistically significant (*, $p<0.05$, $n=4$ sham control and 4 experimental animals).

We also measured the average distance to the active zone of all synaptic vesicles that could be counted per presynaptic terminal and found that it is significantly increased in rats after exposure to anesthesia. In fact, as shown in Fig. 3b, the average distance from the plasma membrane of any given presynaptic vesicle was 280.8 nm±15.8 for GA-treated animals and 224.6±12.8 nm for sham control animals (*, $p<0.05$, $n=4$ sham control and 4 experimental animals). The representative micrographs of sham control (C) and GA-treated (D) neuropil show presynaptic vesicles that are preferentially located further away from the presynaptic plasma membrane (PM) in GA-treated animals compared to sham controls.

Based on the results shown in Figs. 1, 2, and 3, it appears that not only do GA-exposed animals display a decreased number of presynaptic vesicles, but also the spatial organization of their synaptic vesicles is disrupted, with fewer vesicles located in close proximity to the plasma membrane and more vesicles clustered distal to the presynaptic terminal.

Anesthesia Alters the Size of the Readily Releasable Pool of Synaptic Vesicles at Presynaptic Terminals

Given that an early exposure to anesthesia appears to disrupt the spatial organization of synaptic vesicles, we set out to examine whether it can also affect their clustering by changing the size of synaptic vesicle pools within the presynaptic terminals, in particular the size of the readily releasable pool of synaptic vesicles. To address this question, we counted the number of vesicles docked at the active zone in sham control and GA-treated rats and normalized it by the length of the synapse. As shown in Fig. 4a, we found that the number of

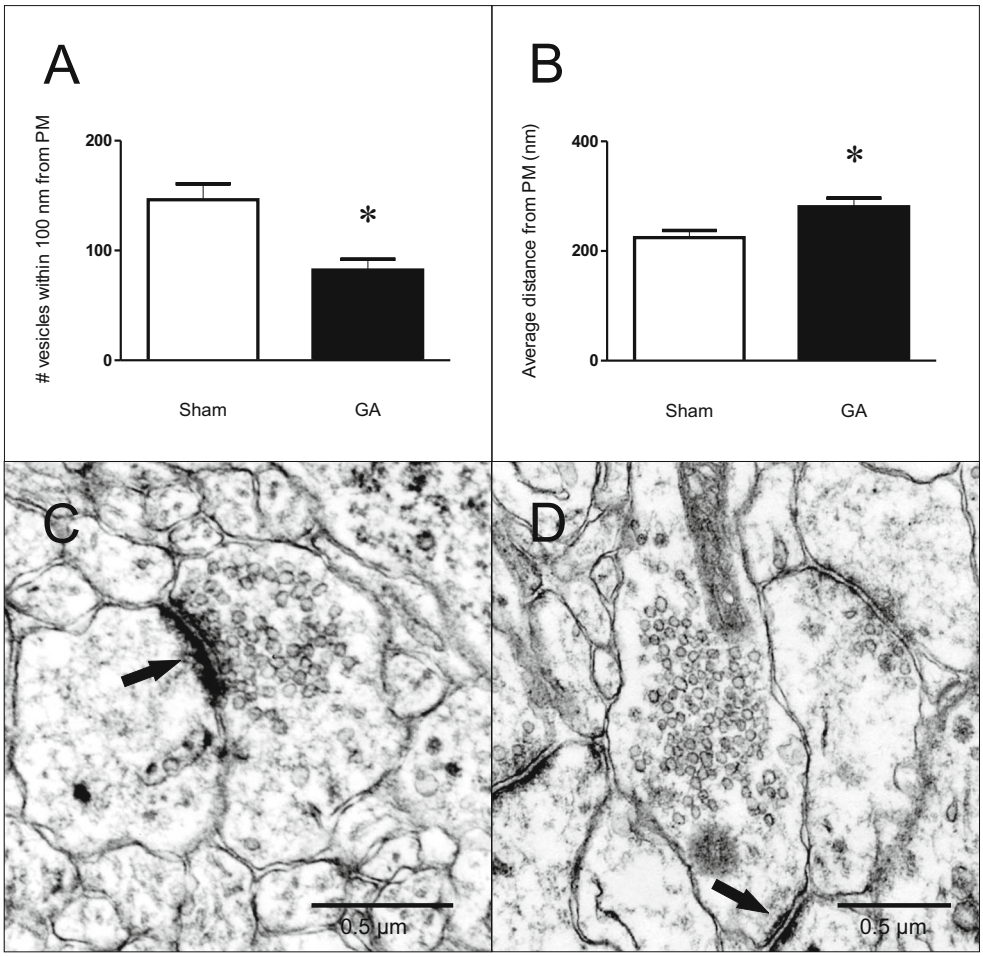

Fig. 3 Exposure to GA disrupts the spatial organization of synaptic vesicles in presynaptic terminals. **a** The number of vesicles located within 100 nm of the active zone is significantly decreased in rats exposed to GA compared to sham controls ($p<0.05$, $n=4$ sham control and 4 experimental animals). **b** Presynaptic vesicles of GA-treated rats are located at an average distance from the active zone that is approximately 20 % higher compared to sham controls ($p<0.05$, $n=4$ sham control and 4 experimental animals). **c**, **d** Representative electron micrographs from a sham control and a GA-exposed animal, respectively. *Arrows* indicate excitatory synaptic contacts. *Calibration bars* 0.5 μm. Note that presynaptic vesicles are preferentially located in the back of the presynaptic terminal, i.e., further away from the presynaptic plasma membrane (PM), in GA-treated animals compared to sham controls

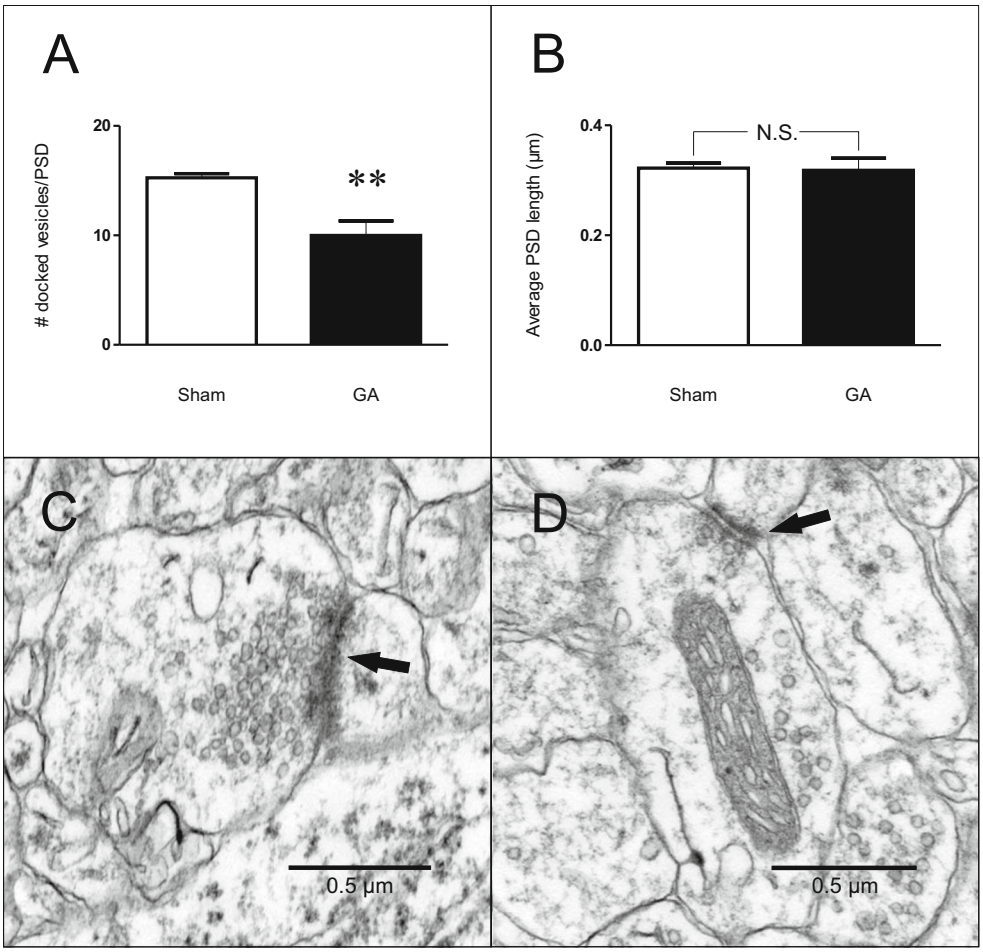

Fig. 4 Anesthesia alters the size of the readily releasable pool of synaptic vesicles at presynaptic terminals. **a** The number of docked vesicles, normalized by the length of the synaptic contact (i.e., length of postsynaptic density), is decreased by more than a third in rats exposed to GA compared to sham controls ($p<0.01$, $n=4$ sham control and 4 experimental animals). **b** There is no difference in the length of postsynaptic densities (PSD) in rats treated with GA compared to sham controls ($p=0.87$, $n=4$ sham controls and 4 experimental rats). **c**, **d** Representative electron micrographs from a sham control and an experimental animal, respectively. *Arrows* indicate synaptic contacts. *Calibration bars* 0.5 μm. Note that the number of synaptic vesicles making contact with the presynaptic plasma membrane is significantly lower in the animal exposed to anesthesia compared to the sham control animal

vesicles contacting the presynaptic membrane was reduced by 35 % in GA-treated rats compared to sham controls (**, $p<0.01$, $n=4$ sham control and 4 experimental rats). The average number of docked vesicles was 9.9±1.3 in animals exposed to GA and 15.2±0.3 in sham controls.

Since the decrease in the number of docked vesicles could be due to an increase in the length of synaptic contacts in GA-treated animals, we compared the length of synaptic contacts in our sham control and GA groups. As shown in Fig. 4b, the length of postsynaptic densities was found to be similar in GA-exposed animals compared to sham controls (0.31± 0.02 nm vs 0.32±0.01 nm, $p=0.87$, $n=4$ sham control and 4 GA-exposed animals). Thus, we conclude that the decrease in the number of vesicles contacting the plasma membrane, rather than the increase in the length of the synaptic contacts, explains the overall decrease in the number of docked vesicles in animals exposed to anesthesia compared to controls. The representative micrographs of sham control (C) and GA-treated (D) neuropils show that the number of synaptic vesicles making contact with the presynaptic plasma membrane is significantly lower in the animal exposed to GA compared to the sham control animal.

Exposure to GA Does Not Affect the Size of Presynaptic Vesicles

We hypothesized that an early exposure to anesthesia may alter the properties of the phospholipid bilayer lining presynaptic vesicles and postulated that such an effect would be possibly reflected by a change in the size of presynaptic vesicles. In order to start to address this hypothesis, we measured the average size and the size distribution of presynaptic vesicles in sham control and GA-treated animals. As shown in Fig. 5a, we found that the average size of synaptic vesicles remained unchanged, 49.7±2.2 nm in sham control animals and 51.5±0.5 nm in GA-treated animals ($p=0.46$, $n=4$ sham control and 4 experimental animals). We also looked at the distribution of the sizes of presynaptic vesicles and found that there were no significant differences in the number of vesicles of any particular diameter between sham control and GA-exposed animals (Fig. 5b). Since it appears that anesthesia does not result in changes in the size of presynaptic vesicles, we propose that an early exposure to anesthesia may not alter the properties of the phospholipid bilayer lining synaptic vesicles at subicular presynaptic terminals.

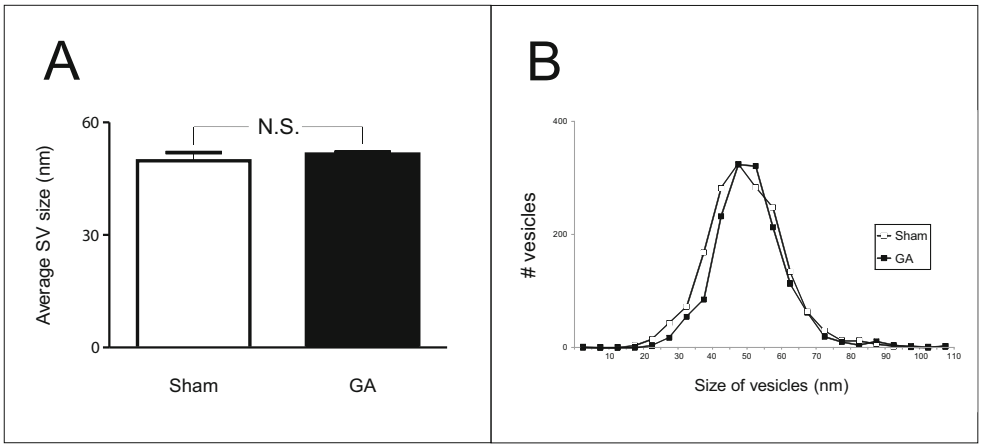

Fig. 5 Exposure to GA does not affect the size of presynaptic vesicles. **a** The average size of presynaptic vesicles (SV) remains unchanged in animals after exposure to GA compared to sham controls (p=0.46, n=4 sham control and 4 experimental animals). **b** The distribution of synaptic vesicle sizes is similar between GA-exposed and sham control animals, with the majority of synaptic vesicle diameters ranging between 35 and 70 nm

Anesthesia Disrupts the Clustering of Synaptic Vesicles at Presynaptic Terminals

Since the decrease in presynaptic vesicle density we found in GA-exposed animals could, at least in part, reflect a change in the degree of tethering of the synaptic vesicles to one another, we measured the distance between each synaptic vesicle and its closest neighbor in subicular terminals from four sham control and four experimental animals. As shown in Fig. 6a, we found that the shortest intervesicle distance was 38.2± 1.8 nm in sham controls and 45.9±2.3 nm in GA-treated animals, and this difference was statistically significant (**, p<0.01). Based on these results, it appears that exposure to anesthesia modulates the ability of synaptic vesicles to "stick" together, causing them to be more loosely associated to one another in GA-exposed animals compared to sham controls.

To examine whether exposure to GA had an effect on synaptic vesicle clustering other than the changes in tethering, we also determined the percentage of presynaptic terminals containing a vesicle cluster. A vesicle cluster was counted every time there was a clear spatial separation of presynaptic vesicles between a pool located in close proximity to the presynaptic membrane and a reserve pool located more distally from the active zone. Upon quantification, we found that 67.1± 12.1 % of presynaptic terminals of sham control animals and 68.0±10.0 % of presynaptic terminals of GA-exposed animals contained a vesicle cluster (p=0.95, n=4 sham control and 4 experimental animals). Thus, we concluded that the proportion of synapses containing vesicle clusters was unchanged after exposure to GA.

Discussion

We report that exposure of immature rats to GA during critical stages of brain development causes significant disruption of the strategic topography of presynaptic vesicles within the nerve

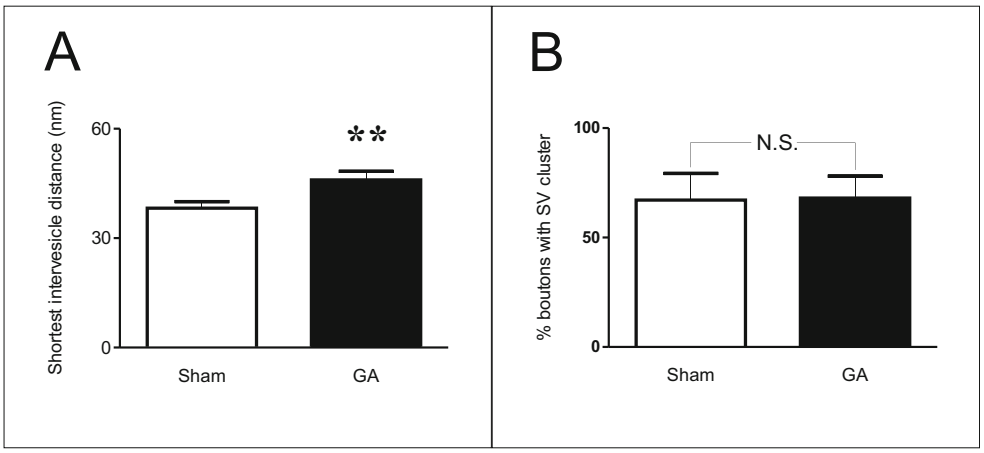

Fig. 6 Anesthesia increases the distance between synaptic vesicles at presynaptic terminals. **a** The average intervesicle distance in presynaptic terminals of GA-exposed rats is increased by approximately 20 % compared to sham controls (p<0.01, n=4 sham control and 4 experimental animals). **b** The percentage of presynaptic terminals containing a vesicle cluster remains unchanged after exposure to GA (p=0.95, n=4 sham controls and 4 experimental animals)

terminals of the subiculum. In addition to a significant decrease in the density of presynaptic vesicles, we observed a reduction of docked vesicles, as well as a reduction of vesicles located within 100 nm from the active zone, in animals 5 days after an initial exposure to GA. We also found that the synaptic vesicles of animals exposed to GA are located more distally with respect to the plasma membrane than those of sham control animals and that the distance between presynaptic vesicles is increased in GA-exposed animals compared to sham controls.

Previously, we have shown that the immature subiculum of rats is extremely vulnerable to anesthesia, i.e., a single exposure to anesthesia results in a permanent deletion of many neurons and synapses [1, 4, 16–19]. Here, we show for the first time that anesthesia-induced damage goes beyond the acute loss of neurons and synapses, leaving numerous surviving synapses with ultrastructural abnormalities indicative of a dysfunctional synaptic vesicle spatial organization. Hence, a single exposure to anesthesia may have far-reaching consequences on the trafficking and spatial organization of vesicles within the synaptic vesicle cycle in nerve terminals in the developing subiculum.

The release of a neurotransmitter into the synaptic cleft is a highly specialized process that requires the coordination of multiple signals and a myriad of proteins within the presynaptic terminals. A tightly organized and finely orchestrated trafficking of synaptic vesicles is fundamental to guaranteeing adequate exchange of information between synapses and is reflected in the precisely regulated topography of presynaptic vesicles within nerve terminals [28]. In fact, it is becoming increasingly evident that the morphological structure of the presynaptic (as well as the postsynaptic) terminal is inseparable from synaptic function and that there is a tight link between the ability of a synapse to maintain an efficient compartmentalization among functionally diverse synaptic vesicle pools and its ability to function properly [28–30]. For example, it is generally accepted that the vesicles docked at the active zone constitute the readily releasable pool of synaptic vesicles, as assessed by electrophysiology, i.e., vesicles that are primed, partially fused to the plasma membrane and ready to be released into the synaptic cleft as soon as calcium enters the nerve terminal [28]. Also, the presence of a strategically positioned reserve pool of synaptic vesicles is pivotal to replenish the readily releasable pool in case of sustained exocytosis. In our study, we found a decrease in the number of vesicles located within 100 nm from the plasma membrane and an overall increase in the distance from the plasma membrane of synaptic vesicles in GA-exposed animals compared to sham controls. Not unexpectedly, the size of the readily releasable pool of vesicles, reflected by the number of docked vesicles, was also found to be decreased in GA-exposed animals. It is reasonable to propose that the latter may be a consequence of the inability to efficiently buffer the readily releasable pool, secondary to a suboptimal localization of the reserve pools, being more distal to the active zone in GA-exposed animals compared to sham controls. Similarly, we believe that the significant increase in the distance between synaptic vesicles in the nerve terminals of GA-exposed animals reflects the inability to maintain a coordinated structure and tether vesicles together in spatially defined pools. Although not within the scope of this study, it is reasonable to propose that the anesthesia-induced perturbation of the synaptic vesicle clustering at subicular nerve terminals may have functional consequences on the efficacy and plasticity of synaptic transmission, and studies are being undertaken to address this hypothesis.

The mechanisms by which GA causes disruption of synaptic vesicle organization in nerve terminals could be multiple. For example, at the level of the presynaptic terminal, the actin cytoskeleton has a crucial role in maintaining and regulating the spatial organization of synaptic vesicles [10–12]. In particular, the actin cytoskeleton is associated with a family of phosphoproteins called synapsins (synapsin I, II, III) which, in turn, are linked to synaptic vesicles [29, 30]. The actin-synapsin scaffold is believed to sequester synaptic vesicles into the reserve pool and when vesicle recycling capacity is exceeded, phosphorylation of synapsins releases the reserve vesicles from the actin-synapsin scaffold, so they can buffer the depletion of the readily releasable pool. Consequently, regulation of the dynamic equilibrium between reserve and readily releasable pools by synapsins underlies important forms of synaptic transmission and plasticity [27–30]. Interestingly, general anesthetics can modulate the state of phosphorylation of synapsins in mouse brain [31] and the impairment of synapsin function has been associated with many behavioral and learning deficits [29, 30]. Several studies have demonstrated reduced numbers of vesicles after synapsin deletion in various types of synapses [32, 33]. Previous studies also suggest that synapsin I and II tether synaptic vesicles into the reserve pool in nerve terminals [27, 32–35]. Accordingly, the decrease in presynaptic vesicle density and the increase in the intervesicle distance we found in GA-treated animals are consistent with the hypothesis of an effect of anesthesia exposure on synapsins, a possible reduction in protein levels, an alteration of phosphorylation status or a disruption of their distribution within presynaptic terminals.

Although synapsins are important candidate targets for GA-induced disruption of synaptic vesicle trafficking, GA may affect synaptic vesicle spatial organization also by directly impairing the actin cytoskeleton. For example, it is known that both isoflurane and propofol neurotoxicity are mediated by actin depolymerization through a BDNF-p75NTR-RhoA cascade [13, 14]. RhoA is a small GTPase that is activated by p75NTR signaling; RhoA activation leads to actin cytoskeleton depolymerization and apoptosis through its effector Rho-associated kinase (ROCK) [5, 13, 15]. Also, a direct regulation of neurotransmitter release at the active zone by actin is

supported by strong experimental evidence. In fact, in hippocampal slices, latrunculin A (a potent toxin which causes actin depolymerization) promotes neurotransmitter release, as demonstrated by an increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs) and the size of evoked EPSCs [12]. Based on the existing evidence that the actin cytoskeleton is critical for synaptic vesicle organization at presynaptic boutons [10–12], that general anesthetics can disrupt actin organization at the presynaptic terminals [13, 15], and that its depolymerization causes perturbations of synaptic transmission [12], it is possible that the impairment of synaptic vesicle spatial organization and trafficking we report herein may be secondary to the disruption of the actin cytoskeleton at the presynaptic terminal, mediated through a BDNF-p75NTR-RhoA-actin signaling cascade. Further studies are needed to examine this notion.

We conclude that anesthesia-induced impairment of synaptogenesis in the immature rat subiculum is marked by significant morphological disturbances of the spatial organization and trafficking of synaptic vesicles in 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 anesthesia.

Acknowledgments Our research is supported by the International Anesthesia Research Society (IARS) Mentored Research Award (to P.I. N.L), the NIH/NICHD HD44517 (to V.J-T.), NIH/NICHD HD44517-S (to V.J-T), Harold Carron endowment (to V.J-T), John E. Fogarty Award TW007423-128322 (to V.J-T), and the National March of Dimes Award (to V.J-T). V.J-T. was an established investigator of the American Heart Association. We are grateful to James Hounshell for writing the Matlab algorithm for X-Y synaptic vesicle clustering analysis, to Nikola Todorovic for helping with vibratome cutting of brains, and to Dr. Christopher M. Sharrow for the valuable comments and careful editing of the manuscript. The authors also wish to thank the Advanced Electron Microscopy Facility, University of Virginia, for the technical assistance with electron microscopy.

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