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Neonatal anesthesia impairs Synapsin 1 and Synaptotagmin 1, two key regulators of synaptic vesicle docking and fusion

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Abstract

Early exposure to anesthetics may interfere with synaptic development and lead to cognitive deficits. We previously demonstrated a decrease in vesicles docked at and within 100 nm from the presynaptic membrane in hippocampal nerve terminals of neonatal rats after anesthesia. Hence, we designed this study to assess the effects of neonatal anesthesia on Synapsin 1 (Syn 1) and Synaptotagmin 1 (Syt 1), two key regulators of vesicle docking and fusion. To test the link between changes in Syn 1 and Syt 1 and behavioral deficits observed after neonatal anesthesia, we also assessed retention memory and fear conditioning in adolescent rats after neonatal anesthesia. Pups received a combination of clinical anesthetics, then Syn 1 and Syt 1 mRNA and protein expression were determined at the peak (postnatal day 8, P8), part-way through (P12) and end of synaptogenesis (P24) in the CA1-subiculum by qPCR and Western Blotting. Anesthesia decreased Syn1 and Syt1 mRNA expression at P8 ($p<0.01$ and $p<0.001$) and P12 ($p=0.001$ and $p=0.017$), but not P24 ($p=0.538$ and $p=0.671$), and impaired Syn1, p-Syn1 and Syt1 protein levels at P8 ($p=0.038$, $p=0.041$ and $p=0.004$, respectively), P12 ($p<0.001$, $p=0.001$ and $p<0.0001$) and P24 ($p=0.025$, $p=0.031$ and $p=0.001$). Anesthetic-challenged rats displayed deficient long-term retention memory ($p=0.019$) and hippocampus-dependent fear conditioning ($p<0.001$). These results suggest that anesthetics alter Syn 1 and Syt 1 during synapse assembly and maturation, raising the possibility that anesthetic interference with Syn 1 and Syt 1 could initiate changes in synaptic function that contribute to the cognitive deficits observed after neonatal anesthesia.

Keywords

synaptic docking; synaptic fusion; vesicle exocytosis; retention memory; freezing behavior

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Introduction:

Approximately 6 million children receive general anesthesia yearly in the United States alone [1]. Animal studies suggest that exposure to anesthetics at a young age is associated with permanent neurocognitive deficits [2–3], however the mechanisms underlying these effects are poorly understood. While it is established that anesthetics modulate synaptic function via presynaptic actions [4,5], the molecular mechanisms underlying anesthetic effects on synaptic vesicle exocytosis remain largely unexplored.

The synapsins are synaptic vesicle-associated proteins that segregate vesicles in a reserve pool, and release them towards the presynaptic plasma membrane for docking upon phosphorylation [6]. Vertebrates possess at least three synapsin genes, of which Synapsin 1 (Syn 1) has the strongest influence on synapse formation and maturation [6]. Synaptotagmin 1 (Syt 1) is the main neuronal sensor that triggers fusion of docked vesicles upon entry of Ca ++ into the nerve terminal [7]. The mammalian brain expresses at least 8 synaptotagmin isoforms, with Syt1 being specialized in fast Ca++-dependent exocytosis.

A previous study from our laboratory demonstrated a reduction in the number of vesicles docked at and within 100 nm from the presynaptic plasma membrane in the CA1-subiculum of anesthesia-treated rats five days after exposure [8]. Hence, the aims of this study were to investigate the effects of neonatal anesthesia on Syn 1 and Syt 1, two key regulators of synaptic vesicle trafficking, docking and fusion, and to test the link between changes in Syn 1 and Syt 1 and the learning and memory deficiencies observed after neonatal anesthesia.

Methods

Animals and Anesthesia:

As described previously [8,9], Sprague-Dawley rats received a single intraperitoneal injection of midazolam (9 mg/kg), followed by 6 hours of nitrous oxide 70%, isoflurane 0.75% and oxygen 30% at the peak of synaptogenesis (P7) [2,8,9]. Controls received an equal volume of intraperitoneal vehicle (0.1% dimethyl sulfoxide), and were kept in a separate chamber at room air for 6 hours. Rats were randomly allocated to anesthesia or control group the morning of the experiment. All rats were maintained at 37 ± 0.5 °C, and gas concentrations were continuously measured with a gas analyzer (Capnomac Ultima, Ohmeda, Madison, WI). At the conclusion of anesthesia, rats were recovered until return of spontaneous movements, and reunited with their mothers. An equal number of male and females was used. Every effort was taken to avoid animals' suffering. All studies were approved by the Institutional Animal Care and Use Committee at the University of Virginia (Charlottesville, VA).

Tissue collection

Brains were harvested at P8, P12 and P24 under brief anesthesia with isoflurane, and sectioned into 700–900 um-thick slices with a vibratome (DTK-1000, Ted Pella, Redding, CA). The CA1-subiculum was collected under a dissecting microscope (10× magnification) according to anatomical maps [10], and snap-frozen in liquid nitrogen for molecular

analysis. Since brain harvesting for molecular studies was a terminal procedure, a separate cohort of animals was used for molecular and behavioral studies. Supplemental Digital Content 1 represents the timeline of the studies.

Western blotting

Proteins were extracted by adding 0.2 ml of ice-cold RIPA buffer [25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS] and 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL)] to the CA1-subiculum tissue. Protein concentration was measured with Pierce Protein Assay Kit (Thermo Scientific, Rockford, IL). Proteins (5 to 20 μg) were loaded in 4–20% Tris-Glycine gels (Mini-PROTEAN TGX, BioRad, Hercules, CA), transferred to nitrocellulose membrane (Millipore, Billerica, MA), blocked with 3% bovine serum albumin at room temperature for 1 h, and incubated overnight at 4 °C with primary antibodies (all used at 1:3000 dilution) against: Syn 1, phosphorylated-Synapsin 1 (p-Syn1, Ser-603), Syt 1 (Cell signaling, Danvers, MA), and anti-glyceraldehyde phosphate dehydrogenase (Gapdh, 1:8000, Sigma-Aldrich, St. Louis, MO). Membranes were washed with TBS-Tween and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000, Cell signaling, Danvers, MA) for 1 h at room temperature. Immunoreactivity was detected using chemiluminescence substrate (SuperSignal West Femto, Thermo Scientific, Rockford, IL), and protein expression was analyzed densitometrically with Gene Tools-Syngene (Synoptics Ltd., Cambridge, England). Protein expression was normalized to Gapdh, and expressed as percent change from control (referred to as 100%).

Quantitative polymerase chain reaction

Total RNA was extracted using RNeasy Mini kit (Qiagen, Germantown, MD). 1 ug RNA was converted to first strand cDNA using iScript cDNA synthesis kit (Bio-RAD, Hercules, CA). The resulting cDNA (2 uL) was subjected to real-time qPCR (Bio-Rad, Hercules, CA) in a final volume of 20 μL, containing 1x SYBR Green supermix (Bio-RAD, Hercules, CA) and 5μM primers (Sigma, St. Louis) to amplify: Syn 1 (Forward: CTGACTAAGACATATGCCAC, Reverse: ACTTGTACCTGTCAGA CATAG); Syt 1 (Forward: GAGAAAGAAGAGCAAGAGAAAC, Reverse: CTTCCAGAATGA CAACAGTC), with Gapdh (qRnoCID00570 18, Bio-RAD, Hercules, CA) as internal control. Amplification conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 40 s. Each sample was run in triplicates, and mean CT values were used to quantify relative fold gene expression by 2^-ΔΔCt method [11]. Fold mRNA changes were expressed relative to control (referred to as 1) after normalization to Gapdh.

Behavioral tests

Barnes Maze—The Barnes maze is a validated test that assesses spatial learning and memory in rodents [12]. It consists of a circular platform with 20 equally spaced holes (SD instruments, San Diego, CA), where one of the holes is connected to a dark chamber (the target box). The test started by placing the rats in the middle of the maze. Aversive noise (85dB) and bright light (200W) shed on the platform were used to encourage rats to find the target box. After training for 4 days, their reference memory was tested on day 5 (short-term

retention) and 12 (long-term retention). Each session was recorded with ANY-maze software (Stoelting Co., Wood Dale, IL). We quantified time to reach the target box, percent of time outside target quadrant, heading angle and path efficiency. Time spent outside of target quadrant was the ratio between total time spent outside of target quadrant and time to reach the target. Heading angle was the angle between the animal's initial direction of movement and a straight line to the target. Path efficiency was calculated by dividing the straight-line distance between first and last animal's position by the total distance traveled (a score of 1 indicated perfect efficiency).

Fear conditioning test

The context-related and tone-related fear conditioning tests reliably assess hippocampal-dependent and non-hippocampal dependent learning, respectively [13]. One day after completion of the Barnes maze, each rat was subjected to 3 tone-foot shock pairings (tone: 2000Hz, 85dB, 30s; foot shock: 1 mA, 2 s) in a test chamber. The following day, each rat was placed in the same chamber, in the absence of tone or shock for 8 min, and freezing behavior was evaluated (context test). Two hours later, the same animal was placed in a triangular-shaped chamber with lemon scent in a bright room; after a 3-min exploratory period, three auditory tones were delivered (2,000 Hz, 85 db) and freezing behavior, i.e., lack of any movement, except for respiration, was observed (tone test).

Data analysis

Western blot and qPCR data were compared with two-tailed unpaired t-test. Behavioral data were analyzed with two-tailed unpaired t-tests, except for training in the Barnes maze, for which two-way repeated measures ANOVA was used. Statistical analysis was performed with GraphPad Prism 7.0 (La Jolla, CA). All values are mean ± S.E.M. P values less than 0.05 were considered statistically significant.

Results

Effects of neonatal anesthesia on Syn 1 and Syt 1

Anesthesia decreased Syn1 mRNA expression compared to control at P8 ($p<0.01$) and P12 ($p=0.001$), but not P24 ($p=0.538$) (Fig.1A). Syn1 protein level was reduced at P8 ($p=0.038$), P12 ($p<0.001$) and P24 ($p=0.025$) (Fig.1B). p-Syn1, the activated form of Syn1 and main effector of docking, was also reduced at P8 ($p=0.041$), P12 ($p=0.001$) and P24 ($p=0.031$) (Fig.1C). The ratio between Syn1 and its phosphorylated fraction was not different between anesthesia-treated and control rats (P8: $p=0.777$; P12: $p=0.514$; P24: $p=0.813$) (Fig.1D). Syt1 mRNA was decreased at P8 ($p<0.001$) and P12 ($p=0.017$), but not P24 ($p=0.671$) (Fig. 1E). Syt1 protein level was impaired at P8 ($p=0.004$), P12 ($p<0.001$) and P24 ($p<0.01$) (Fig. 1F).

Impairment of spatial memory and fear conditioning following neonatal anesthesia

Anesthesia-challenged and control rats trained equally successfully in the task of finding the target box in the Barnes maze (Fig.2A). No difference was found in time to reach target box in the short-term retention test ($p=0.620$). However, anesthesia-challenged rats took longer to reach the target box in the long-term retention test, compared to control ($p=0.019$) (Fig.

2B). Anesthesia-exposed rats spent more time outside the target quadrant (p=0.026, Fig.2C), were more likely to take a heading direction away from the target (p=0.010, Fig.2D), and moved inefficiently to the target box (p=0.043, Fig.2E). Moreover, anesthesia-challenged rats exhibited decreased freezing in the context-related, but not tone-related, fear conditioning test (p<0.001, Fig.2F). Locomotion and coordination were intact following neonatal anesthesia (Fig.3).

Discussion

Our study showed that exposure to anesthesia during a phase of intense synaptic development impairs mRNA and protein expression of Syn1 and Syt1, two presynaptic proteins that are critical for vesicle trafficking, docking and fusion. The results of this study are consistent with our previous ultrastructural observations of a shift in synaptic vesicle localization away from the presynaptic membrane, a reduction in the number of docked vesicles and increased inter-vesicular distance in the nerve terminals of young rats after neonatal anesthesia [8].

Although a number of ion channels and synaptic receptors have been highlighted as potential targets of anesthetics, little is known about the effects of anesthetics on presynaptic vesicle exocytosis. The existing studies are limited by the use of invertebrate organisms or in vitro preparations, therefore may not fully reflect the tissue structure and properties of developed living organisms [4,5,14–16]. In this study we characterized the in vivo expression of Syn 1 and Syt 1at three time points. P8 was chosen to evaluate the acute effects on Syn 1 and Syt 1. P12 was selected in keeping with prior work from our laboratory that documented a reduction in docked vesicles five days after neonatal anesthesia [8]. P24 was chosen to test if altered Syn 1 and Syt 1 expression persists after synaptogenesis, which is complete at three weeks of age in rats [17]. We focused on the CA1-subiculum because this region is important for memory and learning in rats [18].

We found that Syn1 and Syt1 mRNA levels were reduced at P8 and P12, and returned to control levels by P24. Syn1 and Syt1 protein expression was decreased at P8 and P12, and also at P24, despite no changes in mRNA expression at P24, suggesting post-transcriptional modifications of primary transcript mRNA. Since Ca++-calmodulin dependent Kinase II (CAMKII) can activate Syn1 by phosphorylation at Ser-603 [6], we assessed CAMKII activity using antibodies against Syn1 phosphorylated at Ser-603. We found that the ratio between p-Syn 1 and total Syn1 was similar in anesthesia-exposed and control rats at all time points tested. This argues against the notion that anesthetics selectively impair phosphorylation of Syn1, at least through a direct action on CAMKII.

Exposure of neonatal rats to anesthesia resulted in poorer performance on spatial reference memory tasks in the Barnes maze, as indicated by a delay in locating the target box, a greater proportion of time spent outside the target quadrant, a heading direction away from the target, and greater path inefficiency. Rats exposed to anesthesia also had less freezing behavior in the contextual fear conditioning test, suggesting impairment of hippocampus-dependent learning and memory. Interestingly, causal correlation between Syn 1 reduction and spatial memory impairment in the Morris water maze has been demonstrated recently by

Qiao and coworkers [19], implicating Syn 1 as a potential protein substrate for memory formation. Our study showed that anesthesia-induced alteration of Syn 1 and Syt 1 expression in neonatal rats was associated with subsequent memory and learning deficits in juvenile rats. Given the importance of Syn 1 and Syt 1 in synapse assembly and maturation, it is a plausible hypothesis that anesthetic interference with Syn 1 and Syt 1during a phase of intense synaptic development may initiate changes in synaptic function that lead to subsequent memory and learning deficits. Further studies are needed to establish a cause-effect relation between changes in Syn 1 and learning deficits. Our study has limitations. Our anesthesia protocol used several anesthetics in combination, as is done in clinical anesthesia. Therefore, we cannot draw conclusions on the contribution of each agent to the changes in Syn 1 and Syt 1 expression. Further studies with individual anesthetics are needed to clarify their relative importance. Also, given that our anesthetic protocol was delivered to infant rats, it is possible that our findings may only be relevant to the developing brain and remain to be verified in adult animals.

Conclusion

Our study showed that neonatal anesthesia decreased the expression of Syn1 and Syt1, key regulators of synaptic vesicle trafficking, docking and fusion. Impaired Syn1 and Syt1 expression during synaptogenesis was associated with deficits in spatial memory and learning during adolescence, raising the possibility that anesthetic interference with Syn 1 and Syt 1 during a phase of intense synaptic development may disturb synaptic function and contribute to the cognitive disabilities observed after neonatal anesthesia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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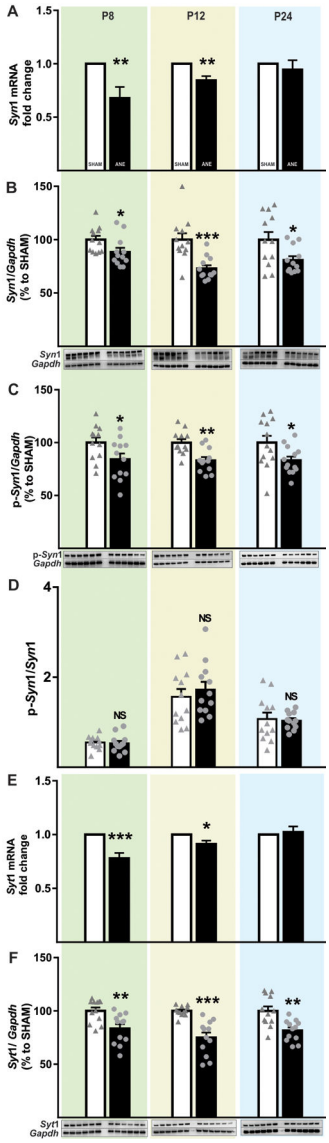

Figure 1. Effects of neonatal anesthesia on Syn 1 and Syt 1.

A: Anesthesia impaired Syn1 mRNA at P8 and P12, but not P24. B, C: Anesthesia decreased Syn1 and p-Syn1 protein levels at P8, P12 and P24 compared to control. D: The ratio between p-Syn 1 and total Syn1 was unchanged in anesthesia-treated rats compared to control. E: Anesthesia impaired Syt1 mRNA at P8 and P12, but not P24. F: Anesthesia decreased Syt1 protein level at P8, P12 and P24. N=7–9 anesthesia and 7–9 control per time point for qPCR. N=12–13 anesthesia and 12–13 control per time point for Western blot.

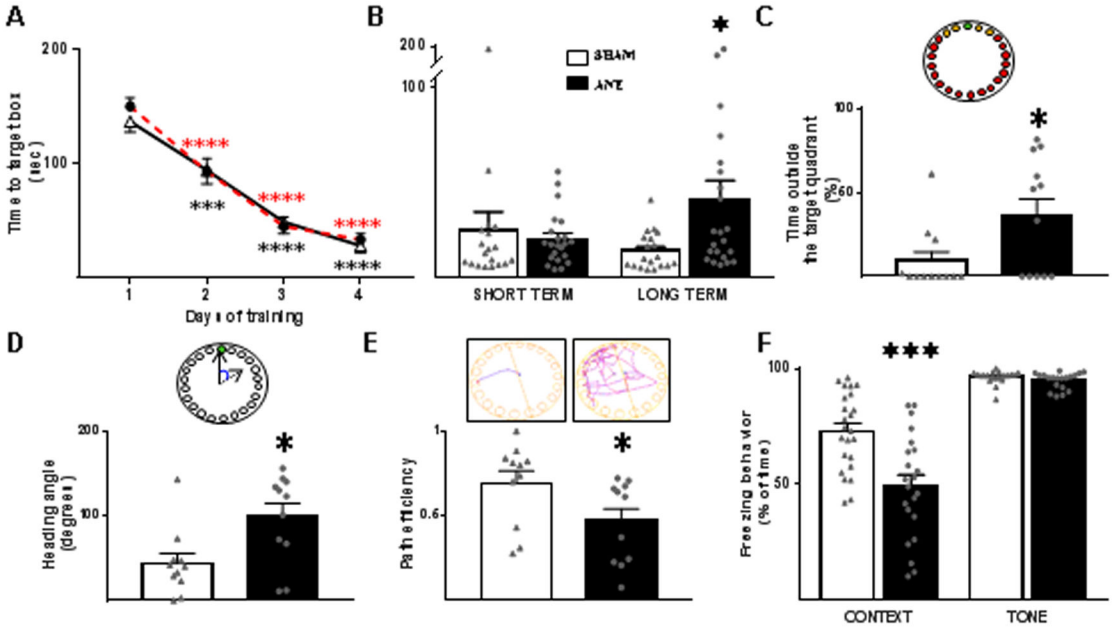

Figure 2. Impairment of spatial memory and fear conditioning following neonatal anesthesia.

A: Rats trained equally successfully in the Barnes maze, as indicated by a shorter time to target box on each training day compared to day 1 (Anesthesia: Day 2: ****, $p<0.0001$; Day 3: ****, $p<0.0001$; Day 4: ****, $p<0.0001$; Control: Day 2: ***, $p<0.001$; Day 3: ****, $p<0.0001$; Day 4: ****, $p<0.0001$). B: There was no difference in time to target box in the short-term retention test between anesthesia-treated and control rats. However, anesthesia-treated rats exhibited delayed target finding in the long term retention test. C: Anesthesia-challenged rats spent more time outside the target quadrant (green and yellow holes) compared to control. D: Anesthesia-treated rats were more likely to take an initial direction away from the target box, as evidenced by a higher heading angle, compared to control. E: Path efficiency was impaired in anesthesia-exposed rats compared to controls. F: Anesthesia-treated rats exhibited decreased freezing in the context-related, but not tone-related ($p=0.138$), fear conditioning test. Panel A, B, C: N=19–22 anesthesia and 19–24 control. Panel D, E, F: N=11–12 anesthesia and 11–12 control.

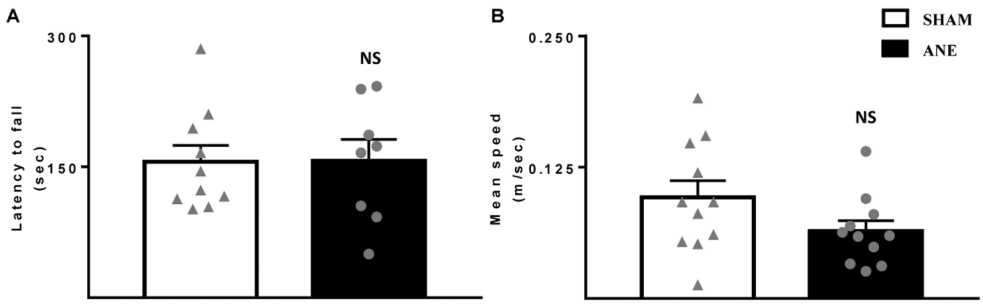

Figure 3. Locomotion and coordination are intact in Syn 1 and Syt 1 deficient rats.

To exclude that poor performance in the Barnes maze and fear conditioning test was due to motor disability, we assessed motor coordination in the rota-rod and mean speed in the Barnes maze. Latency to fall from rota-rod (A) and mean speed in the Barnes maze (B) were not different in anesthesia-treated rats and controls ($p=0.973$ and $p=0.101$) ($N=8$ anesthesia and 10 control).