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Sensitivity to isoflurane anesthesia increases in autism spectrum disorder *Shank3*^{+/-} mutant mouse model

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Introduction

Autism is a heterogeneous developmental disorder characterized by impaired social interaction, impaired communication skills, and restricted and repetitive behavior.^{1–3} The abnormal behaviors of these patients can make their anesthetic and perioperative management difficult.^{4,5} Evidence in the literature suggests that some patients with autism or specific autism spectrum disorders (ASD) exhibit altered responses to pain and to anesthesia or sedation.^{6,7} A better understanding of the biologic reasons for these varied responses to analgesia or anesthesia in these patients may provide a basis for improved clinical management.

One particular ASD, Phelan McDermid Syndrome, has been associated with haplotype mutation or deletion of the molecular scaffolding protein Shank3.⁸ Individuals with this disorder have been anecdotally reported to have a reduced responsiveness to pain and delayed awakening from anesthesia and sedation. A genetic mouse model of Phelan McDermid Syndrome has been developed that has a *Shank3* haplotype truncation (*Shank3*^{+/-}). These mice exhibit important characteristics of autism that mimic human autistic behavior.^{9,10} Shank3 protein is a molecular scaffolding protein essential for synapse

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formation and for mediating N-methyl-D-aspartate receptor (NMDAR)- and metabotropic glutamate receptor (mGluR)-induced excitatory synaptic transmission,^{11–14} potential sites of anesthetic action.^{15–20} We have shown previously that inhalational anesthetic agents can disrupt the interaction of other scaffolding proteins (PSD93 and PSD95) with NMDA and AMPA receptors, resulting in a reduction of the minimum alveolar concentration (MAC) required for anesthesia^{21,22}. Shank binds to PSD95-associated protein GKAP and assembles into a complex of Shank/GKAP/PSD95, coupling NMDAR-PSD95 complexes to regulators of the actin cytoskeleton²³. Shank3 associates with Homer1A and prevents mGluR1-mediated inhibition of NMDAR^{13,24}. *Shank3* haploinsufficiency has been shown to produce deficits in synaptic function and plasticity and decreased AMPA-R expression¹⁰. Further, *shank3* deficiency was shown to reduce surface expression of NR1 subunits and produce NMDA-R hypofunction²⁵. Neurons generated from induced pluripotent stem cells derived from patients with Phelan McDermid syndrome had reduced expression of glutamatergic receptors, decreased synaptic numbers, and defects in excitatory synaptic transmission, which could be reversed by restoring Shank3 expression²⁶. We therefore hypothesized that the *Shank3* haplotype truncation could account for an increased sensitivity to inhalational anesthetics observed in autism.

Isoflurane is an inhalational anesthetic that is often used for patients with mental and neurologic disease who must be anesthetized for surgery or a medical procedure. Patients with mental illness and animal models of mental illness have shown sensitivity changes to anesthetics.^{27,28} Whether functional loss of Shank3 protein can affect the sensitivity of patients to isoflurane is still unknown. This question takes on added clinical relevance in the context of concerns associated with neurotoxicity of anesthetic agents e.g.^{29–31} all of which suggest that reduced anesthetic exposure is likely to be generally desirable, particularly in patients who may be in vulnerable states associated with extremes of age or neurologic disease possibly including autism³². In this study, we examined whether sensitivity to isoflurane anesthesia is altered in mice with a *Shank3* gene mutation.

Methods

Animals

This study was carried out with approval from the Animal Care and Use Committee at Johns Hopkins University and was consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. No surgery was performed, and all efforts were made to minimize animal suffering and reduce the number of animals used.

Shank3^{+/–} mice were provided by Worley's laboratory³³. *Shank3*^{+/–} mice were made on the 129S6/SvEvTac strain and these mice were backcrossed to a C57BL/6J strain for 10+ generations. Male offspring were used for experiments at 6–8 weeks of age. All animals were housed up to 5 per cage on a 14-h light–10 h dark cycle with lights on at 7 am and off at 9 pm. Mice had water and food pellets available ad libitum. All behavioral testing was carried out between 10:00 am and 4:00 pm.

Rotarod test

Using the method described by Mansouri A et al.³⁴ with some modifications, animals were trained on the IITC Rotarod Series 8 rotating rod (IITC Life Science Inc., CA, USA) at a rate of 4 rpm for 60s on two consecutive days before experimental testing was begun. Performance on the rotarod was assessed 1 day before measurement of isoflurane MAC. The rod rotation speed was increased from a rate of 4 rpm to a maximum of 40 rpm in 60 s. The experiment was stopped at a cutoff time of 300s. The duration of time that the mouse remained on the rotating rod was recorded. Each mouse was tested three times at 15-min intervals, and the mean duration on the rod was calculated.

Measurement of isoflurane MAC

The measurement of isoflurane MAC value was carried out as described previously with minor modification.³⁵ Mice were placed in individual Plexiglas chambers, and a rectal temperature probe was inserted under light general anesthesia (1% isoflurane). Each chamber was fitted with a rubber stopper at one end through which the mouse tail and rectal temperature probe protruded. Groups of four mice were given isoflurane in oxygen (100%, 4 L/min total gas flow). A gas sample was continuously drawn, and the anesthetic concentration was measured with an agent analyzer (Ohmeda 5250 RGM, Louisville, CO). The temperature of the mice was kept at 36–38°C with heat lamps throughout the experiment. Mice initially breathed approximately 1.2% isoflurane for 60 min. Then, a 15-cm hemostatic forceps was applied to the tail for 1 min, and the mice were observed for a movement in response to the stimulation. Motor activity (gross movement of the head, extremities, and/or body) was considered a positive response. If the mouse exhibited a response, the anesthetic concentration was increased by 0.1%; if no response was observed, the concentration was decreased by 0.1%. After 20 min of equilibration, the tail was stimulated again. Only the middle third of the tail was used for tail clamping, and the clamp was always placed proximal to the previous test site. The anesthetic concentration was increased (or decreased) in steps of 0.1% until the positive response disappeared (or appeared if it was initially absent). No mortality was observed in mice during the procedure. MAC was defined as the concentration midway between the highest concentration that permitted movement in response to the stimulus and the lowest concentration that prevented movement.

Determination of isoflurane righting reflex EC₅₀ (RREC₅₀)

After we measured MAC, we reduced the isoflurane concentration in half for 20 min and turned the animal onto its back to test the righting reflex, defined as a return onto all four paws within 1 minute.^{20–22} The isoflurane concentration was reduced by 0.1% for 20 min if the animal did not right itself, and the righting reflex was subsequently retested. RREC₅₀ was calculated for each mouse as the mean value of the anesthetic concentrations that just permitted and just prevented the righting reflex.

Western blotting

Shank3^{+/-} mice and WT mice were sacrificed by cervical dislocation (n=6), and the caudal brain and spinal cord (lumbar) were harvested. Mice used for biochemistry were from a

different cohort than those used in behavioral tests. Caudal brain was grossly dissected in a mouse brain slice mold at approximately -6.00 to -7.00 mm Bregma and -7.00 to -7.70 mm Bregma. Vestibular nuclei were then block dissected from the two brain slices. Total proteins from these tissues were extracted. The tissues were then homogenized in homogenization buffer (10mM Tris-HCl, 5mM MgCl_2 , 2mM EGTA, 1mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM pepstatinA, and 320mM sucrose [pH 7.4]). The crude homogenates were centrifuged at 700g for 15 min at 4°C. Then the supernatants were combined and diluted in resuspension buffer (10mM Tris-HCl, 5mM MgCl_2 , 2mM EGTA, 1mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM pepstatin A, and 250mM sucrose [pH 7.4]). Next, the protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. The membranes were blocked in 0.1% Tween-20 in Tris-HCl-buffered saline (TBST) containing 5% nonfat milk for 1h at room temperature and then immunoblotted with primary antibodies(anti-shank3: 1:1000, Santa Cruz, Dallas, Texas; anti-Homer1b/c: 1:500, Santa Cruz; anti-NR1: 1:1000, Millipore, Billerica, MA; anti-NR2A/B: 1:2000, Millipore; anti-GKAP: 1:500, NeuroMab, Davis, CA; anti-PSD95: 1:1000, NeuroMab; anti-mGluR1: 1:1000, Cell-signaling, Danvers, MA; anti- mGluR5: 1:5000, Cell-Signaling; β -actin: 1:100,000, Sigma-Aldrich, St. Louis, MO) in TBST buffer containing 5% nonfat milk overnight at 4°C. After being washed extensively in TBST, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (Bio-Rad Laboratories, Hercules, CA) at a dilution of 1:2,000. Proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ). β -actin served as a loading control. The immunoblotting bands were quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD) and analyzed.

Statistical Analysis

Statistical analysis was carried out by Student's t-test with Graphpad Prism version 6.05 software (Graphpad Inc, La Jolla, CA). Post-hoc power analysis for $\alpha = 0.05$ was conducted using an online freeware (www.clinicalcalc.com). Data are expressed as mean $\pm$ standard deviation (SD), and statistical significance was set at $P < 0.05$. All statistical analysis output is shown in supplemental figure 1 along with a graphical representation of the raw data.

Results

Shank3^{+/-} C mice display normal motor function in rotarod test

Rotarod testing was performed to assess motor function in *shank3*^{+/-} C mice. The mean length of time that the *Shank3*^{+/-} C mice remained on the rotarod was not significantly different from that of the WT mice (*Shank3*^{+/-} C mice: 24.69 $\pm$ 3.87 s; WT mice: 23.78 $\pm$ 2.61 s; n=10, $P=0.54$, Fig. 1). Thus, the *Shank3*^{+/-} C mice did not have any inherent motor dysfunction.

Isoflurane MAC and RREC₅₀ are decreased in shank3^{+/-} C mice

Both MAC and RREC₅₀ values were significantly lower in *shank3*^{+/-} C mice than in WT mice (Table 1). Isoflurane MAC was 1.30 $\pm$ 0.08% in *shank3*^{+/-} C mice and 1.49 $\pm$ 0.10% in

WT mice (n = 10/group, $P < 0.01$). Isoflurane RREC₅₀ was $0.73 \pm 0.08\%$ in *shank3*^{+/-} C mice and $0.93 \pm 0.08\%$ in WT mice (n = 10/group, $P < 0.01$).

Protein expression in Shank3 signaling pathway in caudal brain of WT and *Shank3*^{+/-} C mice

Western blot analysis showed that Shank3 protein was down regulated in the caudal brain of *shank3*^{+/-} C mice compared to that in WT mice. (*Shank3*^{+/-} C mice: 0.696 ± 0.115 ; WT mice: 1.37 ± 0.063 ; n=6, $P < 0.01$, Fig. 2A, B) In addition, NMDAR1 (NR1) expression also was lower in *Shank3*^{+/-} C mice than in WT mice (*Shank3*^{+/-} C mice: 1.74 ± 0.164 ; WT mice: 2.58 ± 0.152 ; n=6, $P < 0.01$). However, NR2A/B, PSD95, GKAP, Homer1b/c, mGluR1, and mGluR5 were not significantly different between the two genotypes ($P > 0.05$, Fig. 2C, D).

Protein expression in Shank3 signaling pathway in spinal cord of WT and *Shank3*^{+/-} C mice

Western blot analysis of spinal cord homogenate showed that Shank3 was down regulated in *Shank3*^{+/-} C mice compared to that in WT mice (*Shank3*^{+/-} C mice: 0.886 ± 0.076 ; WT mice: 1.27 ± 0.053 ; n=6, $P < 0.01$, Fig. 3A, B). PSD95 expression also was significantly lower in *Shank3*^{+/-} C mice than in WT mice (*Shank3*^{+/-} C mice: 0.93 ± 0.029 ; WT mice: 1.23 ± 0.054 ; n=6, $P < 0.01$). However, no significant difference was observed in the expression of NR1, NR2A/B, GKAP, Homer1b/c, mGluR1, or mGluR5 ($P > 0.05$, Fig. 3C, D).

Discussion

In this study, we found that *Shank3*^{+/-} C mice are more sensitive to isoflurane anesthesia than are WT mice. *Shank3*^{+/-} C mice had a lower MAC and lower RREC₅₀ of isoflurane than did WT mice, as well as reduced expression of some proteins of the Shank3 signaling pathway in the caudal brain and spinal cord. These differences suggest that changes in shank3 related signaling pathways and protein expression contribute to the decrease in isoflurane MAC and RREC₅₀.

Recently, several animal models have been developed to simulate the characteristics of autism.³⁶ *Shank3* is one of the best-characterized genes implicated in ASD.^{36–38} *Shank3*^{+/-} C mutant mice display abnormal social behaviors that mimic ASD-like symptoms.^{33,39} Wang et al.⁴⁰ reported that the *shank3*(e4-9) homozygous mutant mouse exhibited impaired motor ability. However, we did not observe any motor dysfunction in the *Shank3*^{+/-} C mice that we tested on the rotarod. It is possible that the homozygous mutation imparts a motor disability that is prevented when one normal copy of the gene is present, as in the heterozygous mice that we used. We used the haplotype truncation for Shank3 because it mimics the human haplotype alteration of Shank3 in Phelan McDermid Syndrome. It was important in our study that motor dysfunction not confound the results of our tests of MAC and RREC₅₀.

Clinical studies have shown that patients with ASD or language communication obstacles have different anesthetic requirements than those without such disabilities, suggesting that such patients may need additional monitoring during anesthesia.^{7,41–43} A study by Asahi et al.⁴² showed that for dental procedures, autistic patients have greater requirements for propofol, an intravenous anesthetic, than do intellectually impaired patients. However, the mechanisms of action of propofol and inhalational anesthetics are substantially different. In

our study, we found that MAC and RREC₅₀ of isoflurane in the *Shank3*^{+/-} C mice were lower than those of the control mice, indicating that genetic alterations in *Shank3* may increase isoflurane sensitivity. The most immediate clinical question raised by our study is whether we may be routinely over-anesthetizing autistic patients with this mutation. Given that autistic patients often receive premedication with midazolam and/or ketamine in addition to volatile anesthetics⁴⁴ perhaps the use of a BIS monitor, which has previously been suggested as a helpful adjunct in the care of patients with ASD⁴⁴, would be useful in avoiding excessive anesthetic exposure. The goal of such an intervention would be at a minimum to reduce delayed awakenings and side effects of volatile anesthetics and furthermore to reduce the extent of exposure of this vulnerable set of patients to drugs that are potential neurotoxins. Whether such an intervention might generalize beyond the limited population of autistic patients with the *Shank3* or similar mutations is an unanswered question that could be addressed with further study.

The striking change in righting reflex that we found is strongly correlated with MAC-awake, and as such is potentially highly relevant to the practice of anesthesia in autistic individuals, at least in those with *Shank3* or other similar mutations. MAC-awake is defined as the minimum alveolar concentration at which a patient can emerge from anesthesia upon verbal stimulation at the time of awakening⁴⁵. Consolidation of memories is not believed to occur at this depth of anesthesia, making it a critical value in clinical practice⁴⁶. It is difficult to know how to model key points in human anesthetic depth using animal models. MAC varies greatly between rodents and humans, which may to some extent reflect a difference in metabolic activity, but may also reflect differences in how movement is mediated in the central nervous system⁴⁷. The righting reflex has been identified as the parameter most closely related to hypnosis and unconsciousness in humans⁴⁷.

The finding that a transgenic mouse which is a model for autism has enhanced sensitivity to isoflurane raises important questions about the relationship between anesthetics and autism. First is the question of whether autism might enhance the sensitivity of the developing brain to pediatric anesthetic neurotoxicity (PAN). No data currently exist beyond what are presented in this manuscript to directly address this question, but it has been speculated that anesthetic disruption of the function of the PDZ domain, which is an integral part of *Shank* function, may contribute to PAN⁴⁸, and it is possible that these effects are exacerbated by *shank* mutation. Further investigation of this hypothesis will require testing of learning and memory in *Shank3*^{+/-} C mutant mice exposed to anesthesia in the early postnatal period. The second question of interest is whether anesthetic exposure at early postnatal ages may enhance the risk of autism or exacerbate symptoms of this disorder. The clinical literature addressing this question has yielded mixed results. In a large study of the New York State Medicaid database DiMaggio and co-workers found that exposure to anesthesia and surgery correlated with an increase in billing codes for learning disorders, including autism, but there is no subgroup analysis available to examine autism alone^{49,50}. However, two birth cohort studies failed to show any association between early anesthesia exposure and later diagnosis of autism^{51,52}. Here again, the *Shank3*^{+/-} C mutant mouse model could provide valuable insight, it provides the opportunity to test whether an animal with propensity towards autism displays either an increased rate of autism phenotype or an increase in the frequency or intensity of behavioral manifestations with anesthetic exposure.

Isoflurane inhibits the release of glutamic acid from nerve terminals^{53–59} and excitability of glutamate receptors in the postsynaptic membrane.^{60–62} Isoflurane anesthesia has been suggested to occur partly through acting on glutamate and GABA receptors.⁶³ Recent studies have shown that noncompetitive NMDAR antagonists can increase the anesthetic potency of the inhalational anesthetic halothane.^{64,65} In addition, isoflurane anesthesia was shown to down regulate the phosphorylation of NR1 at Ser897 in hippocampus and striatum.⁶⁶ Furthermore, mice with a heterozygous point mutation at Asn598 of the NR1 subunit showed impaired righting reflex.⁶⁷ Previous studies have focused mainly on whole brain to observe the relationship between NMDARs and righting reflex.^{4,5,35,67,68} However, the righting reflex involves the vestibular nuclei located in the caudal brain. Therefore, in our study, we focused mainly on the expression of NMDAR in caudal brain. We found that NR1 expression was down regulated in *Shank3*^{+/-} C mice compared with that in WT mice, suggesting that down regulated NR1 in caudal brain contributes to loss of RREC₅₀ in these mice.

The activity of NMDA receptors was regulated by the Shank3 signaling pathway, mGluR-homer-shank3-GKAP-PSD95.^{23,69–72} PSD95, a scaffolding protein in the Shank3 signaling pathway, is an important molecule in NMDA receptor-mediated signal transmission.^{73,74} Knocking down PSD95⁷⁵ and disrupting NMDAR-PSD95 interactions³⁵ in spinal cord can reduce isoflurane MAC. Our data showed that the expression of PSD95, but not that of NMDAR, is reduced in spinal cord of *Shank3*^{+/-} C mice, suggesting that down regulated PSD95 in spinal cord contributes to the decrease of isoflurane MAC in *shank3*^{+/-} C mice.

We found that expression levels of GKAP, homer1, mGluR1, mGluR5, and NR2A/B were unaltered in caudal brain and spinal cord of *Shank3*^{+/-} C mice. However, in an in vitro study by Verpelli et al.,¹³ knockdown of Shank3 by siRNA caused a decrease in mGluR5 in cultured rat hippocampal and cortical neurons; the authors did not examine protein expression in vivo. In another study, postsynaptic density proteins were altered to varying degrees in hippocampus and striatum of *Shank3* mutant mice,⁴⁰ but that mouse model was different from the one we used. In yet another *Shank3* mutant mouse model (*ex13-16*^{-/-}), Peca et al.⁷⁶ found that homer1 and GKAP, but not PSD95, were reduced in the striatum. These findings indicate that protein expression in the Shank3 signaling pathway depends on the brain region and on the Shank3 mutation.

In conclusion, our study demonstrates that a *Shank3*^{+/-} C mutation in mice causes down regulation of NR1 in caudal brain and PSD95 in spinal cord. Decreased expression of NR1 and PSD95 in the central nervous system of *Shank3*^{+/-} C mice could reduce MAC and RREC₅₀ of isoflurane. However, this phenomenon needs confirmation in a clinical study. If Shank3 mutations are found to affect anesthetic sensitivity in patients with ASD, better communication and stricter monitoring of anesthetic depth may be necessary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. The effects of anesthetics on a mouse with the Shank3+/- C mutation, which is a genetic model of autism, is studied.
2. The mutation is associated with a reduction in both the MAC and RREC50 of isoflurane.
3. Western blotting shows downregulation of NR1 in vestibular nuclei and PSD95 in spinal cord.
4. These findings suggest that Shank3+/- C confers enhanced sensitivity to isoflurane.

Fig. 1. *Shank3*^{+/-ΔC} mice have no functional motor impairment

Performance on the rotarod test was not significantly different between the *Shank3*^{+/-ΔC} mice and WT mice. Data are shown as mean ± SD. (n=10/group, *P*=0.54).

Fig. 2. Expression of proteins in Shank3 signaling pathway in caudal brain of WT and *Shank3*^{+/-ΔC} mice

A: Representative Western blot showing expression of shank3 in caudal brain. Expression of β-actin was used as an internal reference. **B:** Quantification of band density shows that Shank3 expression was significantly lower in *Shank3*^{+/-ΔC} mice than in WT mice. **P*<0.01; n=6/group. **C:** Representative Western blot showing expression of NR1, NR2A/B, PSD95, GKAP, Homer1b/c, mGluR1, and mGluR5 in caudal brain. **D:** Quantification of band densities shows that only NR1 expression was down regulated in the caudal brain of *Shank3*^{+/-ΔC} mice compared to that in WT mice. **P*<0.01; n=6/group.

Fig. 3. Expression of proteins in Shank3 signaling pathway in spinal cord of WT and *Shank3*^{+/-ΔC} mice

A: Representative Western blot showing expression of Shank3 in spinal cord. Expression of β-actin was used as an internal reference. **B:** Quantification of band density shows that Shank3 expression was significantly lower in *Shank3*^{+/-ΔC} mice than in WT mice.**P*<0.01;n=6/group. **C:** Representative Western blot showing expression of NR1, NR2A/B, PSD95, GKAP, Homer1b/c, mGluR1, and mGluR5 in spinal cord. **D:** Quantification of band densities shows that only PSD95 expression was down regulated in the spinal cord of *Shank3*^{+/-ΔC} mice compared to that in WT mice.**P*<0.01;n=6/group.

Table 1MAC and RREC50 of isoflurane in WT and *Shank3*^{+/-} C mice

	WT mice	<i>shank3</i> ^{+/-} C mice	F	P
MAC(%)	1.49±0.10	1.30±0.08 *	1.3	<0.01
RREC ₅₀ (%)	0.93±0.08	0.73±0.08 *	1.0	<0.01

Data are displayed as mean ± standard deviation (n=10).

* Significant differences compared with WT mice.