Neurotoxicology and Teratology 60 (2017) 69–74

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

Sensitivity to isoflurane anesthesia increases in autism spectrum disorder *Shank3*$^{+/\Delta c}$ mutant mouse model

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ARTICLE INFO

Article history:
Received 3 July 2016
Received in revised form 14 October 2016
Accepted 9 November 2016
Available online 14 November 2016

Keywords:
Autism Spectrum Disorder
Shank3
Isoflurane
NR1
PSD95

ABSTRACT

Autism is a heterogeneous developmental disorder characterized by impaired social interaction, impaired communication skills, and restricted and repetitive behavior. The abnormal behaviors of these patients can make their anesthetic and perioperative management difficult. 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. A genetic mouse model of one particular ASD, Phelan McDermid Syndrome, has been developed that has a Shank3 haplotype truncation (*Shank3*$^{+/\Delta c}$). These mice exhibit important characteristics of autism that mimic human autistic behavior. Our study demonstrates that a *Shank3*$^{+/\Delta C}$ mutation in mice is associated with a reduction in both the MAC and RREC50 of isoflurane and down regulation of NR1 in vestibular nuclei and PSD95 in spinal cord. Decreased expression of NR1 and PSD95 in the central nervous system of *Shank3*$^{+/\Delta C}$ mice could help reduce the MAC and RREC50 of isoflurane, which would warrant 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.

1. Introduction

Autism is a heterogeneous developmental disorder characterized by impaired social interaction, impaired communication skills, and restricted and repetitive behavior (Lord et al., 2000a, 2000b; Association, A.P., 1994). The abnormal behaviors of these patients can make their anesthetic and perioperative management difficult (van der Walt and Moran, 2001; Bagshaw, 2011). 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 (Allely, 2013; Capp et al., 2010). 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 (Betancur and Buxbaum, 2013). 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*$^{+/\Delta c}$). These mice exhibit important characteristics of autism that mimic human autistic behavior (Bangash et al., 2011; Bozdagi et al., 2010). Shank3 protein is a molecular scaffolding protein essential for synapse formation and for mediating *N*-methyl-D-aspartate receptor (NMDAR)- and metabotropic glutamate receptor (mGluR)-induced excitatory synaptic transmission (Roussignol et al., 2005; Uchino et al., 2006; Verpelli et al., 2011; Freche et al., 2012) potential sites of anesthetic action (Sou et al., 2006; Sharko and Hodge, 2008; McFarlane et al., 1992; Daniell, 1992; Brosnan and Thiesen, 2012; Ishizaki et al., 1999). 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 (Fang et al., 2003; Tao et al., 2015). 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 cytoskeleton (Naisbitt et al., 1999). Shank3 associates with Homer1A and prevents mGluR1-mediated inhibition of NMDAR (Verpelli et al., 2011; Sala et al., 2005). Shank3

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http://dx.doi.org/10.1016/j.ntt.2016.11.002
0892-0362/

haploinsufficiency has been shown to produce deficits in synaptic function and plasticity and decreased AMPA-R expression (Bozdagi et al., 2010). Further, Shank3 deficiency was shown to reduce surface expression of NR1 subunits and produce NMDA-R hypofunction (Duffney et al., 2013). Neurons generated from induced pleuripotent 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 expression (Shcheglovitov et al., 2013). 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 (Anon, 1994; Eckel et al., 2010). 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. (Rappaport et al., 2015; Brown and Purdon, 2013; Woldegerima et al., 2016) 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 autism (Chien et al., 2015). In this study, we examined whether sensitivity to isoflurane anesthesia is altered in mice with a *Shank3* gene mutation.

2. Methods

2.1. 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$^{+/\Delta c}$ mice were provided by Worley's laboratory (Kouser et al., 2013). *Shank3*$^{+/\Delta c}$ 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.

2.2. Rotarod test

Using the method described by Mansouri et al. (2012) 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 300 s. 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.

2.3. Measurement of isoflurane MAC

The measurement of isoflurane MAC value was carried out as described previously with minor modification (Tao and Johns, 2008). 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.

2.4. Determination of isoflurane righting reflex EC_{50} ($RREC_{50}$)

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 min (Ishizaki et al., 1999; Fang et al., 2003; Tao et al., 2015). 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. $RREC_{50}$ was calculated for each mouse as the mean value of the anesthetic concentrations that just permitted and just prevented the righting reflex.

2.5. Western blotting

Shank3$^{+/\Delta c}$ 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 (10 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM pepstatin A, and 320 mM sucrose [pH 7.4]). The crude homogenates were centrifuged at 700*g* for 15 min at 4 °C. Then the supernatants were combined and diluted in resuspension buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM pepstatin A, and 250 mM 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 1 h 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 1:2000. Proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ). β-Actin served as a loading control. The