ANESTHESIOLOGY

Mitochondrial Function in Astrocytes Is Essential for Normal Emergence from Anesthesia in Mice

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- In mice, restriction of loss of the mitochondrial complex I gene *Ndufs4* to glutamatergic neurons confers a profound hypersensitivity to volatile anesthetics.
- Astrocytes are crucial to glutamatergic synapse functioning during excitatory transmission.

What This Article Tells Us That Is New

- In a tamoxifen-activated astrocyte-specific *Ndufs4(KO)* mouse, the induction EC50s for tail clamp in both isoflurane and halothane were similar between the control and astrocyte-specific *Ndufs4(KO)* mice at 3 weeks after 4-hydroxy tamoxifen injection. However, the emergent concentrations in both anesthetics for the astrocyte-specific *Ndufs4(KO)* mice were half that of the controls.
- Similarly, the induction EC50s for loss of righting reflex were similar between the control and astrocyte-specific *Ndufs4(KO)* mice; concentrations for regain of righting reflex in both anesthetics for the astrocyte-specific *Ndufs4(KO)* mice were much less than the control.
- Thus, mitochondrial complex I function within astrocytes is essential for normal emergence from anesthesia.

ABSTRACT

Background: In mice, restriction of loss of the mitochondrial complex I gene *Ndufs4* to glutamatergic neurons confers a profound hypersensitivity to volatile anesthetics similar to that seen with global genetic knockout of *Ndufs4*. Astrocytes are crucial to glutamatergic synapse functioning during excitatory transmission. Therefore, the authors examined the role of astrocytes in the anesthetic hypersensitivity of *Ndufs4(KO)*.

Methods: A tamoxifen-activated astrocyte-specific *Ndufs4(KO)* mouse was constructed. The specificity of the astrocyte-specific inducible model was confirmed by using the green fluorescent protein reporter line *Ai6*. Approximately 120 astrocyte-specific knockout and control mice were used for the experiments. Mice were anesthetized with varying concentrations of isoflurane or halothane; loss of righting reflex and response to a tail clamp were determined and quantified as the induction and emergence EC50s. Because norepinephrine has been implicated in emergence from anesthesia and astrocytes respond to norepinephrine to release gliotransmitters, the authors measured norepinephrine levels in the brains of control and knockout *Ndufs4* animals.

Results: The induction EC50s for tail clamp in both isoflurane and halothane were similar between the control and astrocyte-specific *Ndufs4(KO)* mice at 3 weeks after 4-hydroxy tamoxifen injection (induction concentration, EC50(ind)—isoflurane: control = 1.27 ± 0.12, astrocyte-specific knockout = 1.21 ± 0.18, $P = 0.495$; halothane: control = 1.28 ± 0.05, astrocyte-specific knockout = 1.20 ± 0.05, $P = 0.017$). However, the emergent concentrations in both anesthetics for the astrocyte-specific *Ndufs4(KO)* mice were less than the controls for tail clamp; (emergence concentration, EC50(em)—isoflurane: control = 1.18 ± 0.10, astrocyte-specific knockout = 0.67 ± 0.11, $P < 0.0001$; halothane: control = 1.08 ± 0.09, astrocyte-specific knockout = 0.59 ± 0.12, $P < 0.0001$). The induction EC50s for loss of righting reflex were also similar between the control and astrocyte-specific *Ndufs4(KO)* mice (EC50(ind)—isoflurane: control = 1.02 ± 0.10, astrocyte-specific knockout = 0.97 ± 0.06, $P = 0.264$; halothane: control = 1.03 ± 0.05, astrocyte-specific knockout = 0.99 ± 0.08, $P = 0.207$). The emergent concentrations for loss of righting reflex in both anesthetics for the astrocyte-specific *Ndufs4(KO)* mice were less than the control (EC50(em)—isoflurane: control = 1.0 ± 0.07, astrocyte-specific knockout = 0.62 ± 0.12, $P < 0.0001$; halothane: control = 1.0 ± 0.04, astrocyte-specific KO = 0.64 ± 0.09, $P < 0.0001$); N ≥ 6 for control and astrocyte-specific *Ndufs4(KO)* mice. For all tests, similar results were seen at 7 weeks after 4-hydroxy tamoxifen injection. The total norepinephrine content of the brain in global or astrocyte-specific *Ndufs4(KO)* mice was unchanged compared to control mice.

Conclusions: The only phenotype of the astrocyte-specific *Ndufs4(KO)* mouse was a specific impairment in emergence from volatile anesthetic-induced general anesthesia. The authors conclude that normal mitochondrial function within astrocytes is essential for emergence from anesthesia.

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The mechanism and identities of cells in the central nervous system that contribute to the anesthetic response are not well elucidated. Inhibition of mitochondrial complex I function has been proposed as a possible molecular

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mechanism of action of volatile anesthetics.[1,2] Both the *Caenorhabditis elegans* mutant *gas-1* and the *Drosophila* mutant ND23, each defective in a single distinct mitochondrial complex I subunit, display increased sensitivity to volatile anesthetics.[1,3] In addition, clinical studies show that some children with complex I defects are hypersensitive to sevoflurane.[4] Similarly, a mouse model of complex I dysfunction, *Ndufs4(KO)*, is hypersensitive to volatile anesthetics.[5] Furthermore, the function of complex I is inhibited by volatile anesthetics at concentrations that match the whole animal EC50s of normal and mutant mice.[1,2] Thus, mitochondrial complex I function profoundly affects anesthetic sensitivity across the animal kingdom.

In the mouse, restriction of *Ndufs4* loss to glutamatergic (VGLUT2-expressing) neurons conferred the same profound hypersensitivity to volatile anesthetics as seen with global loss of the protein, with an EC50 one third that of wild-type mice.[6] This finding implicates a role for glutamatergic synaptic transmission in mediating volatile anesthetic hypersensitivity. Glutamatergic synapses have been shown to consist of three cells: a presynaptic neuron, a postsynaptic neuron, and a supporting astrocyte.[7] The configuration is termed the tripartite synapse,[7] where the astrocytic roles include both glutamate reuptake[8,9] and modulation of synaptic transmission.[10] We therefore questioned whether astrocytic function contributes to the change in anesthetic sensitivity in *Ndufs4(KO)*.

In this study, we have investigated the role of astrocytes in mediating the effect of volatile anesthetics by constructing a conditional loss of *Ndufs4* in astrocytes. Because this animal loses the gene acutely during adulthood and only in astrocytes, no compensatory changes are expected during development, leading to confounding phenotypes. The resulting astrocyte-specific *Ndufs4(KO)* animal was tested for responses to isoflurane and halothane using two different anesthetic endpoints: loss of righting reflex and response to a tail clamp. We hypothesized that the astrocyte-specific *Ndufs4(KO)* would be hypersensitive to the volatile anesthetics when compared to control mice.

Materials and Methods

Generation of Astrocyte-specific *Ndufs4(KO)* Mice

All studies were approved by the Institutional Animal Care and Use Committee of the Seattle Children's Research Institute. Mice with a conditional exon 2-floxed allele of the *Ndufs4* gene ($Ndufs4^{lox/lox}$) were a kind gift from the Palmiter laboratory at the University of Washington. The 4-hydroxy tamoxifen–inducible Cre-recombinase expressing line *Pgfap* (*glial fibrillary acidic protein*)–*CreERT2* was purchased from the Jackson Laboratory (USA; Jax stock no. 012849). *Pgfap-CreERT2* mice were crossed to $Ndufs4^{\Delta/+}$ mice following the breeding scheme in figure 1A. Offspring that were *Pgfap-CreERT2*/+ and heterozygous for the *Ndufs4* deletion (Δ/+) were selected and crossed to *Ndufs4* mice floxed at exon 2 to create *Pgfap-CreERT2*/+;$Ndufs4^{\Delta/lox}$ (conditional knockouts of *Ndufs4*) and *Pgfap-CreERT2*/+;$Ndufs4^{+/lox}$ mice (controls). The *Pgfap* promoter–driven Cre-*ERT2* fusion enzyme requires induction by 4-hydroxy tamoxifen for nuclear translocation and function.[11] 4-Hydroxy tamoxifen (Sigma, USA) was diluted to a final concentration of 10 μg/μl in autoclaved, filtered sunflower seed oil and stored at −20°C until administration. The inducible knockout and control mice were injected intraperitoneally with 4-hydroxy tamoxifen at a dose of 50 μg/g bodyweight daily for a week beginning at postnatal day 33 and tested for anesthetic behavior in isoflurane and halothane at 3 and 7 weeks after injections. Two mice of approximately 120 injected mice did not survive the injection regime and were excluded from the analyses.

To demonstrate 4-hydroxy tamoxifen–induced exon excision by the Cre-recombinase, *Pgfap-CreERT2* mice were crossed to $Ndufs4^{lox/lox}$ mice following the breeding scheme in figure 1B. *Pgfap-CreERT2*/+;$Ndufs4^{lox/+}$ offspring were selected from the offspring and crossed to $Ndufs4^{lox/lox}$ to generate *Pgfap-CreERT2*/+;$Ndufs4^{lox/lox}$ mice. After intraperitoneal injection with 4-hydroxy tamoxifen, the deletion was confirmed by polymerase chain reaction.

Generation of Astrocyte-specific *Ai6* Reporter Mice

The inducible Cre-recombinase expressing line, *Pgfap-CreERT2*/+, was crossed to the Ai6 (*ai6/ai6*) reporter line,[12] which produces ZsGreen, a green fluorescent protein only in Cre-recombinase expressing cells. When injected with 4-hydroxy tamoxifen, the astrocytes of the *Pgfap-CreERT2*/+;*ai6*/+ progeny express Cre-recombinase and ZsGreen. The efficiency of Cre-recombinase induction and recombination by 4-hydroxy tamoxifen was assessed by immunohistochemistry (see the Immunohistochemical Analysis section below).

Behavioral Testing

The mice (six or seven for each experiment; the exact number is specified in the figure legends) were anesthetized with isoflurane or halothane and assayed for loss of righting reflex or response to a tail clamp as described by Sonner *et al.*[13] The volatile anesthetics were delivered by inline gas lines to the mouse chamber. Step sizes were 0.1% for both volatile anesthetics in loss of righting reflex, 0.2% in tail clamp. Equilibration time was 10, 20, or 30 min at each step, for separate sets of mice (data shown for 20-min interval experiments only). For induction and emergence assays (done consecutively), the same step sizes and equilibration

times were maintained. Anesthetic concentrations were analyzed by gas chromatography as described.[14] The animals were kept warm on a heating pad throughout and allowed to recover for at least 24 h before testing again, with a different anesthetic or for a different endpoint. The observers were blinded to the genotype of the tested mice. The concentration for induction of anesthesia was defined as the average concentrations of gas in the last sample before loss of response and in the first sample at which response was lost. The concentration for emergence was defined as the average concentration of gas in the first sample at which the animal once again responded and the sample immediately prior. All EC50s for induction and emergence were calculated from the quantal endpoints, *i.e.* the averages of the induction or emergent concentrations for all the mice in a specific anesthetic. The mice were tested at 3 and 7 weeks after injection of 4-hydroxy tamoxifen and then sacrificed. All animals survived the anesthetic exposure.

Immunohistochemical Analysis

The mice were euthanized using CO_2, and isolated brains were fixed in 4% paraformaldehyde overnight at 4°C, cryoprotected in 30% sucrose in phosphate-buffered saline for 3 days, and embedded in optimal cutting temperature compound (Tissue-tek, Sakura, USA). The brains were then sliced at 10-μm thickness and mounted on slides. For heat-induced epitope retrieval, the slides were then boiled in sodium citrate buffer (pH = 6.0) at 100°C for 20 min using a water bath. After this treatment, Cre-recombinase–mediated ZsGreen fluorescence was quenched but recaptured with anti-green fluorescent protein antibodies. The slides were then blocked in 10% normal donkey serum in phosphate-buffered saline for 1 h at room temperature. The primary antibodies used were mouse anti-glial fibrillary acidic protein (1:100, Chemicon, USA) and goat anti-green fluorescent protein (1:500, Abcam, USA) incubated overnight. The secondary antibodies used were donkey anti-mouse IgG–Alexa Fluor 568 (1:1,000, Abcam) and donkey anti-goat IgG–Alexa Fluor 488 (1:2,000, Life Technologies, Inc., USA) and were incubated for 1 hr at room temperature.

Norepinephrine Assay

Brains of 40-day-old global *Ndufs4(KO)* and wild-type mice along with those of 60-day-old astrocyte-specific knockout mice (3 weeks after 4-hydroxy tamoxifen injection) were snap-frozen in dry ice and stored at −80°C. The 60-day old astrocyte-specific knockout animals were used to allow for complete loss of the NDUFS4 protein after 4-hydroxy tamoxifen injection at postnatal day 33.[15] The brains were thawed, homogenized in PBS-containing protease inhibitor cocktail (Sigma), sonicated on ice, and centrifuged. After estimating the protein content of the supernatant, 20 μg each of the total protein extract was subjected to norepinephrine extraction and measurement following the manufacturer's protocol (mouse/rat norepinephrine assay kit, catalogue no. NOU39-K010, Eagle Biosciences, USA).

Statistics

The effective concentration for 50% of the animals tested (EC50) for volatile anesthetics was determined as described by Sonner *et al.*,[13] using an up-and-down method. Values for EC50s were compared between the wild type and knockout strains using two-tailed two-sample *t* tests with unequal variance. No statistical power calculation was conducted before the study. The sample sizes were based on the sample sizes necessary to establish significance in previous studies with global and glutamatergic-specific knockouts of *Ndufs4*.[6] For the norepinephrine assay, the norepinephrine concentrations were compared between the wild type, global *Ndufs4(KO)*, and astrocyte-specific *Ndufs4(KO)* brain samples using one-way ANOVA and assessed by constructing a bar plot. We set the *P*-value threshold at $P < 0.01$ to lessen the likelihood of reporting a false-positive result. Because we compared multiple groups, a Bonferroni correction was performed to determine significance of the *P* values. Significance was defined as a $P < 0.01$ for all analyses except where subjected to a Bonferroni correction where the critical $P < 0.01$ was divided by the number of comparisons to derive the corrected cutoff. The measures of variability assessed by SD are indicated in the figures and legends. Outliers, if any, were always included in the analyses. All statistical analyses were performed using R version 3.4.1 and plotted using ggplot2.

Results

Characterization of the Inducible *Pgfap-CreERT2* Model

We constructed the inducible astrocyte-specific *Ndufs4(KO)* mouse model, with the Cre-recombinase expressed under the control of an astrocyte-specific promoter *Pgfap* (fig. 1A). Activation of Cre-recombinase was induced by 4-hydroxy tamoxifen injections at postnatal days 33 to 40, after *gfap* expression had ended in neuronal progenitors, thereby temporally limiting the expression and nuclear localization of Cre-recombinase to astrocytic cells. Knockout of *Ndufs4* in the brain cells of 4-hydroxy tamoxifen–injected *Ndufs4*$^{lox/lox}$ mice was confirmed by genotyping the mice brains with and without 4-hydroxy tamoxifen injections (fig. 1B). At 3 and 10 days after injection, we found that Cre-recombinase–mediated excision of *Ndufs4* was evident in the brains of the 4-hydroxy tamoxifen–injected *Pgfap-CreERT2/+;Ndufs4*$^{lox/lox}$ mice, whereas it was absent in the brains of sham-injected *Pgfap-CreERT2/+;Ndufs4*$^{lox/lox}$ mice (fig. 1B). The *lox* and knockout (Δ) allelic polymerase chain reaction amplification bands in the gel from the astrocyte-specific *Ndufs4(KO)*

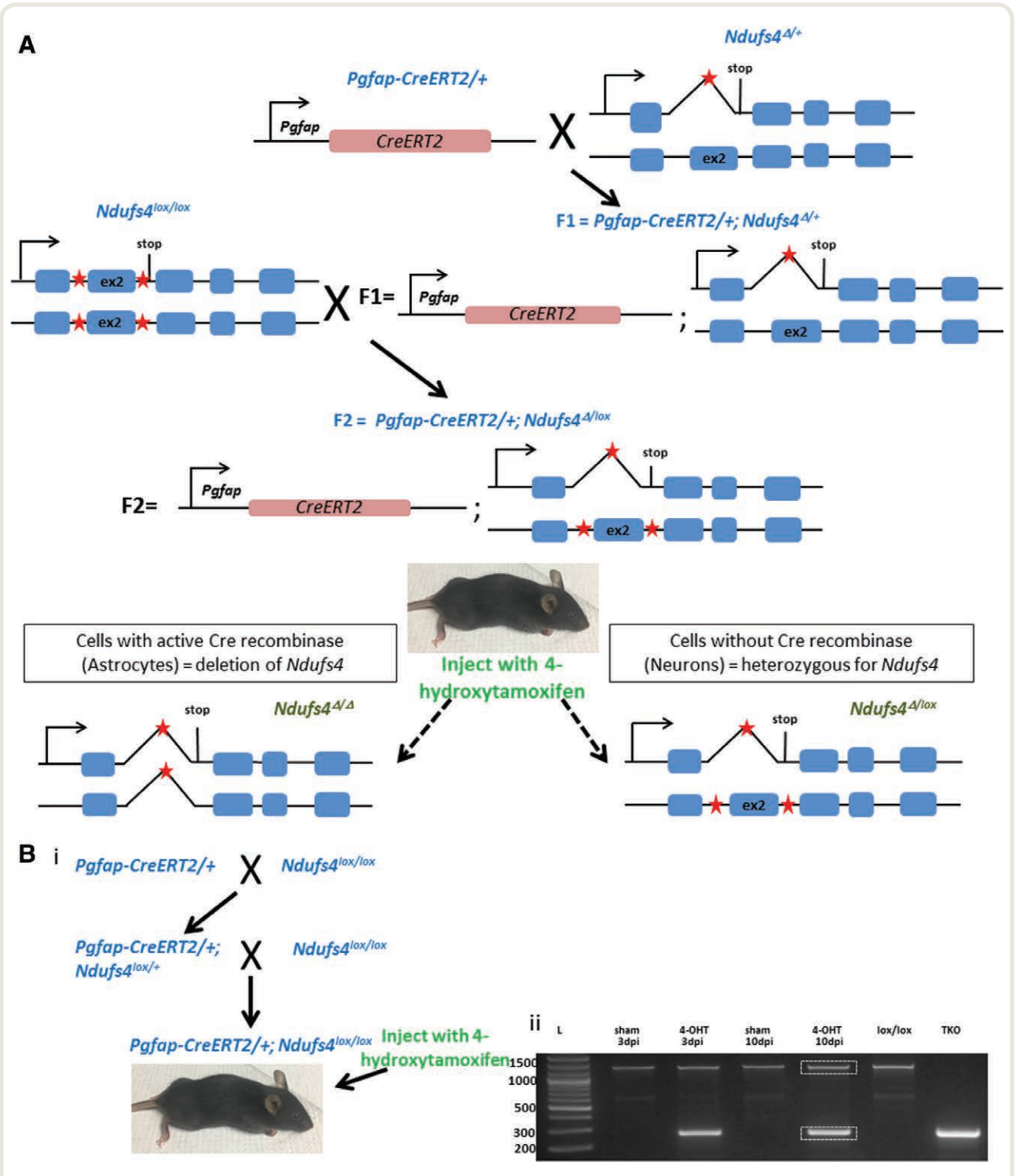

Fig. 1. Generation of the astrocyte-specific *Ndufs4(KO)* mouse model. (*A*) Schematic showing the crossing strategy used. The heterozygous *Ndufs4*$^{\Delta/+}$ mice do not display hypersensitivity to volatile anesthetics or the Leigh syndrome pathogenesis displayed by the global KO, indicating that *Ndufs4* is haplo-sufficient. The *Pgfap-CreERT2* allele has a dominant phenotype; the breeding recommendation from the JAX lab for these mice is to maintain them as heterozygotes. *Pgfap-CreERT2/+* mice were crossed to *Ndufs4*$^{\Delta/+}$ mice. From the resulting F1, *Pgfap-CreERT 2/+;Ndufs4*$^{\Delta/+}$ offspring were selected and crossed to *Ndufs4*$^{lox/lox}$ mice to generate F2 *Pgfap-CreERT2/+;Ndufs4*$^{\Delta/lox}$ (conditional astrocyte-specific *Ndufs4(KO)*) and *Pgfap-CreERT2/+;Ndufs4*$^{+/lox}$ mice (controls). We used *Ndufs4*$^{lox/\Delta}$ instead of *Ndufs4*$^{lox/lox}$ to avoid possible recombination caused by the leakiness of the promoter (as seen with the *Pvglut2*-driven Cre mice). Both genotypes were injected with 4-hydroxytamoxifen intraperitoneally at a concentration of 50 μg/g bodyweight daily from postnatal days 33 to 39. *Red stars* indicate (continued)

Fig. 1. (continued) the *loxP* sites. (*B*, *i*) Schematic of generation of mice to confirm loss of full-length *Ndufs4* upon 4-hydroxy tamoxifen–induced activation of Cre-recombinase in astrocytes. *Pgfap-CreERT2/+* mice were crossed to *Ndufs4*$^{lox/lox}$ mice; from the progeny, *Pgfap-CreERT2/+;Ndufs4*$^{lox/+}$ animals were selected by genotyping and crossed again with *Ndufs4*$^{lox/lox}$ to generate *Pgfap-CreERT2/+;Ndufs4*$^{lox/lox}$ mice, which were injected with 4-hydroxy tamoxifen or vehicle control (sham). The brains of the injected mice were harvested after 3 and 10 days postinjection (dpi) and genotyped. (*B*, *ii*) Genotyping gel of brains of *Pgfap-CreERT2/+;Ndufs4*$^{lox/lox}$ after 4-hydroxy tamoxifen or sham injections, using polymerase chain reaction (*lanes 2* to *5*). dpi, days postinjection of 4-hydroxy tamoxifen or sham oil control). Sham injections yield only a large uncut band at 1,300 bp, whereas 4-hydroxy tamoxifen injections show both the large band (uncut in neurons) and a smaller band (excised in astrocytes) at 270 bp. Sequencing of excised bands outlined in *white rectangles* (*lane 5*), confirmed a full-length allele as well as a truncated allele lacking the second exon, as predicted by size, in the 4-hydroxy tamoxifen–injected mice. Genomic DNA isolated from the tails of *Ndufs4*$^{lox/lox}$ mice and total *Ndufs4(KO)* (TKO) were used as controls (*lanes 6* and *7*, respectively). *Lane 1* shows the DNA ladder (L) size markers (base pairs). 4-OHT, 4-hydroxy tamoxifen.

were excised and sequenced to confirm Cre-recombinase–mediated deletion of exon 2. This result confirmed that 4-hydroxy tamoxifen injections resulted in knockout of *Ndufs4* in the brain cells. The astrocyte-specific KO mouse was similar to the controls in terms of life span, appearance, grooming characteristics, and growth pattern. Unlike the global *Ndufs4(KO)*, the astrocyte-specific KO mice did not become progressively ataxic, lose weight, or die young.

The NDUFS4 antibody did not resolve astrocyte-specific loss of the protein in a background in which all other cells in the central nervous system (CNS) were fully expressing it. Therefore, to determine the specificity of the 4-hydroxy tamoxifen–induced Cre-lox system, we crossed the 4-hydroxy tamoxifen–sensitive *Pgfap-CreERT2* driver line with the *ai6/ai6* reporter mice,[12] which harbors a Cre-inducible green fluorescent protein (ZsGreen) expression cassette. After genotyping the progeny, *Pgfap-CreERT2/+;ai6/+* mice and control *ai6/+* sibling mice were injected daily with 4-hydroxy tamoxifen for a week from postnatal days 33 to 40. When injected with 4-hydroxy tamoxifen, the glial fibrillary acidic protein expressing astrocytes of *Pgfap-CreERT2/+;ai6/+* mice should express induced ZsGreen. Three weeks after the last day of injections, the brains were harvested and immunostained (fig. 2; supplemental fig. 1, http://links.lww.com/ALN/B820). Specific expression of Cre-recombinase within astrocytes of the *Cre/+;ai6/+* mice was confirmed by the yellow appearance of the colocalized glial fibrillary acidic protein (labeled *red*) and ZsGreen (labeled *green*) under confocal microscopy (fig. 2). The control *ai6/+* brain slices did not coexpress ZsGreen, and hence the astrocytes appeared red.

Anesthetic Sensitivity

Tail Clamp. The half-life for the NDUFS4 protein in mouse brain has been shown to be 17 days; its half-life in astrocytes is not known.[15] To allow for possible tissue-specific differences in protein half-life, we tested the mice at 3 and 7 weeks after knocking out the gene; the phenotype remained stable over time. We subjected the mice to 10-, 20-, and 30-min intervals of anesthetic exposure at each step to rule out a pharmacokinetic effect. All data are shown for 20 min of exposure of mice to each concentration of the volatile anesthetic. The astrocyte-specific *Ndufs4(KO)* (*Pgfap-creERT2/+;Ndufs4*$^{\Delta/lox}$) and control sibling mice were first tested for tail clamp response at 3 and 7 weeks after injection of 4-hydroxy tamoxifen, with increasing concentrations of isoflurane or halothane. The induction concentrations for the loss of tail flick were not different between the astrocyte-specific *Ndufs4(KO)* and control animals in both isoflurane and halothane (induction concentration, EC50(ind)—isoflurane: control = 1.27 ± 0.12, astrocyte-specific *Ndufs4(KO)* = 1.21 ± 0.18, P = 0.495; halothane: control = 1.28 ± 0.05, astrocyte-specific *Ndufs4(KO)* = 1.20 ± 0.05, P = 0.017 at 3 weeks; isoflurane: control =1.24 ± 0.07, astrocyte-specific *Ndufs4(KO)* = 1.18 ± 0.13, P = 0.305; halothane: control = 1.19 ± 0.04, astrocyte-specific *Ndufs4(KO)* = 1.22 ± 0.07, P = 0.396 at 7 weeks; table 1). Control animals emerged from the anesthetized states at concentrations similar to their induction concentrations. However, the conditional astrocyte-specific *Ndufs4(KO)*s emerged from the anesthetic state at significantly lower concentrations than did the controls (emergence concentration, EC50(em)—isoflurane: control =1.18 ± 0.10, astrocyte-specific *Ndufs4(KO)* = 0.67 ± 0.11, $P < 0.0001$; halothane: control = 1.08 ± 0.09, astrocyte-specific *Ndufs4(KO)* = 0.59 ± 0.12, $P < 0.0001$ at 3 weeks; isoflurane: control = 1.20 ± 0.05, astrocyte-specific *Ndufs4(KO)* = 0.67 ± 0.10, $P < 0.0001$; halothane: control = 1.15 ± 0.10, astrocyte-specific *Ndufs4(KO)* = 0.66 ± 0.10, $P < 0.0001$ at 7 weeks; table 1; fig. 3; supplemental fig. 2, http://links.lww.com/ALN/B821). Although the difference between the induction/emergence concentrations was not as large as seen with the astrocyte-specific *Ndufs4(KO)*, there was a statistically significant change between the induction *versus* emergence in control animals in halothane, with 1.28% *versus* 1.08%, respectively, at 3 weeks ($P = 5.695 \times 10^{-4}$). The difference was not seen at 7 weeks (1.19% *vs.* 1.15%, P = 0.277).

Loss of Righting Reflex. To determine whether the altered concentration for emergence was specific to a behavioral endpoint and possibly a CNS region, we tested a second

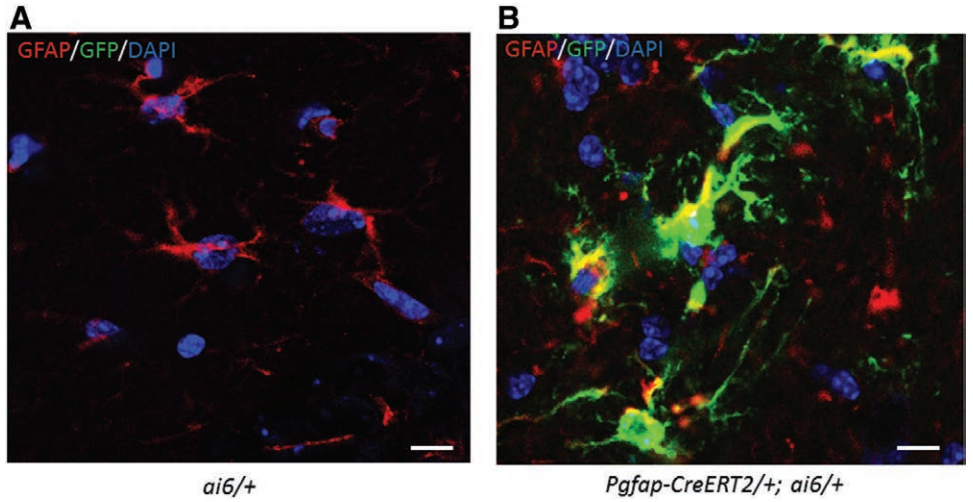

Fig. 2. Confirmation of the specificity of the Cre-lox system by immunohistochemistry of the Ai6 reporter line. Confocal images of the 4-hydroxy tamoxifen–injected *Pgfap-CreERT2/+;ai6/+* and *ai6/+* mice brain slices. (*A*) *ai6/+* mice that only express a *green* marker (ZsGreen) when activated by the Cre-recombinase were used as controls. Nuclei were stained with 4[prime],6[prime]-diamino-2-phenylindole (DAPI, *blue*), astrocytes with anti-glial fibrillary acidic protein (GFAP, *red*), and ZsGreen with anti-green fluorescent protein (GFP, *green*). There is no *green* seen in astrocytes because there is no Cre-recombinase acting in these animals. (*B*) Immunohistochemistry using anti-glial fibrillary acidic protein antibody reveals the colocalization of ZsGreen in the astrocytes of *Pgfap-CreERT2/+;ai6/+* reporter mice when Cre-recombinase is activated by 4-hydroxy tamoxifen. Thus, the localization of ZsGreen marks those cells with *Pgfap*-driven Cre-recombinase–mediated excision of the floxed gene.

Table 1. Effects of Astrocyte-specific *Ndufs4(KO)* on Anesthetic Sensitivity for Response to Tail Clamp

	Tail Clamp Emerge/Induce							
	Isoflurane				Halothane			
	Control		Astrocyte-specific *Ndufs4(KO)*		Control		Astrocyte-specific *Ndufs4(KO)*	
No. of weeks	Induction	Emergence	Induction	Emergence	Induction	Emergence	Induction	Emergence
3	1.27 ± 0.12	1.18 ± 0.10	1.21 ± 0.18	0.67 ± 0.11*	1.28 ± 0.05	1.08 ± 0.09	1.20 ± 0.05	0.59 ± 0.12*
7	1.24 ± 0.07	1.20 ± 0.05	1.18 ± 0.13	0.67 ± 0.10*	1.19 ± 0.04	1.15 ± 0.10	1.22 ± 0.07	0.66 ± 0.10*

The tables show the EC50s for emergence and induction for isoflurane or halothane for control mice or astrocyte-specific *Ndufs4(KO)* at 3 and 7 weeks after 4-hydroxytamoxifen injections. The endpoint used is response to tail clamp. The numbers in each cell are the measured values for the EC50s (emergence) and the EC50s (induction), respectively. *P* values compare the knockout emergence/induction ratio to the corresponding control ratio. N = 6 for controls; N ≥ 6 for the astrocytic *Ndufs4(KO)*.

*$P < 0.005$.

endpoint, loss of righting reflex. The anesthetic concentration requirement for loss of righting reflex (induction) was similar between the control and astrocyte-specific KO mice in isoflurane and halothane (induction concentration, EC50(ind)—isoflurane: control = 1.02 ± 0.10, astrocyte-specific *Ndufs4(KO)* = 0.97 ± 0.06, $P = 0.264$; halothane: control = 1.03 ± 0.05, astrocyte-specific *Ndufs4(KO)* = 0.99 ± 0.08, $P = 0.207$ at 3 weeks; isoflurane: control = 0.92 ± 0.07, astrocyte-specific *Ndufs4(KO)* = 0.98 ± 0.05, $P = 0.128$; halothane: control = 0.98 ± 0.08, astrocyte-specific *Ndufs4(KO)* = 0.93 ± 0.13, $P = 0.410$ at 7 weeks; table 2). The control animals regained the righting reflex (emergence) at close to the induction concentration, whereas the astrocyte-specific *Ndufs4(KO)*s recovered their righting reflex at significantly lower concentrations than the controls (emergence concentration, EC50(em)—isoflurane: control = 1.0 ± 0.07, astrocyte-specific *Ndufs4(KO)* = 0.62 ± 0.12, $P < 0.0001$; halothane: control = 1.0 ± 0.04, astrocyte-specific *Ndufs4(KO)* = 0.64 ± 0.09, $P < 0.0001$ at 3 weeks; isoflurane: control = 0.89 ± 0.06, astrocyte-specific *Ndufs4(KO)* = 0.68 ± 0.08, $P = 0.0005$; halothane: control = 0.94 ± 0.04, astrocyte-specific *Ndufs4(KO)* = 0.55 ± 0.07, $P < 0.0001$ at 7 weeks; table 2; fig. 4; supplemental fig. 2, http://links.lww.com/ALN/B821). The differences

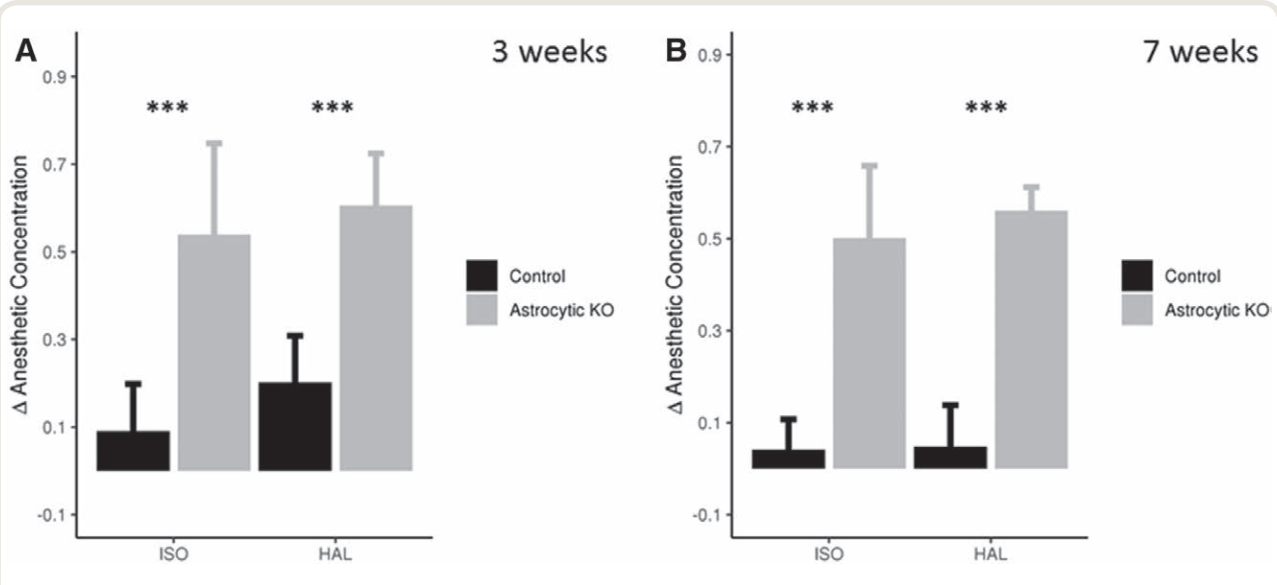

Fig. 3. Astrocyte-specific knockout (KO) of *Ndufs4* causes hysteresis between induction and emergence in tail clamp. (*A*) The change in the anesthetic concentration (Δ Anesthetic Concentration) between induction and emergence required to respond to tail clamp before and after exposure to isoflurane (ISO) or halothane (HAL), at 3 weeks postinjection of 4-hydroxy tamoxifen. N = 6 for control; N = 7 for the astrocyte-specific *Ndufs4(KO)*. (*B*) The change in the anesthetic concentration required to respond to tail clamp before and after exposure to isoflurane or halothane at 7 weeks postinjection of 4-hydroxy tamoxifen. N = 6 for control and the astrocyte-specific *Ndufs4(KO)*. Tests for significance were done on the difference between the EC50s for induction and emergence: ***$P < 0.001$. The *error bars* represent SD.

Table 2. Effects of Astrocyte-specific *Ndufs4(KO)* on Anesthetic Sensitivity for Loss of Righting Reflex

	Loss of Righting Reflex Emerge/Induce							
	Isoflurane				Halothane			
	Control		Astrocyte-specific *Ndufs4(KO)*		Control		Astrocyte-specific *Ndufs4(KO)*	
No. of Weeks	Induction	Emergence	Induction	Emergence	Induction	Emergence	Induction	Emergence
3 weeks	1.02 ± 0.10	1.0 ± 0.07	0.97 ± 0.06	0.62 ± 0.12*	1.03 ± 0.05	1.0 ± 0.04	0.99 ± 0.08	0.64 ± 0.09*
7 weeks	0.92 ± 0.07	0.89 ± 0.06	0.98 ± 0.05	0.68 ± 0.08*	0.98 ± 0.08	0.94 ± 0.04	0.93 ± 0.13	0.55 ± 0.07*

The table shows the EC50s for emergence and induction for isoflurane or halothane, for control mice or astrocyte-specific *Ndufs4(KO)* at 3 and 7 weeks after 4-hydroxytamoxifen injections. The endpoint used is loss of righting reflex. The numbers in each cell are the measured values for the EC50s (emergence) and the EC50s (induction), respectively. *P* values compare the knockout emergence/induction ratio to the corresponding control ratio. N = 6 for controls; N ≥ 6 for the astrocytic *Ndufs4(KO)*.

*$P < 0.005$.

between induction and emergence seen for both loss of righting reflex and tail clamp were similar for halothane and isoflurane and were not altered by prolonged exposure times (30 min) at each concentration of the anesthetic.

Norepinephrine Assay

Because norepinephrine is known to activate cortical astrocytes,[16] we tested whether the levels of norepinephrine in the global *Ndufs4(KO)* are reduced when compared with the astrocyte-specific *Ndufs4(KO)* or control mice. There was no significant difference in the total norepinephrine content in whole brain extracts between the global or astrocyte-specific *Ndufs4* KOs, compared with wild-type mice (fig. 5).

Discussion

Astrocytes affect synaptic transmission by multiple mechanisms. During synaptic transmission, glutamate is removed from the synaptic cleft by astrocytes, actively converted to glutamine by the astrocyte-specific glutamine synthetase, and supplied back to neurons through glial transporters SN1

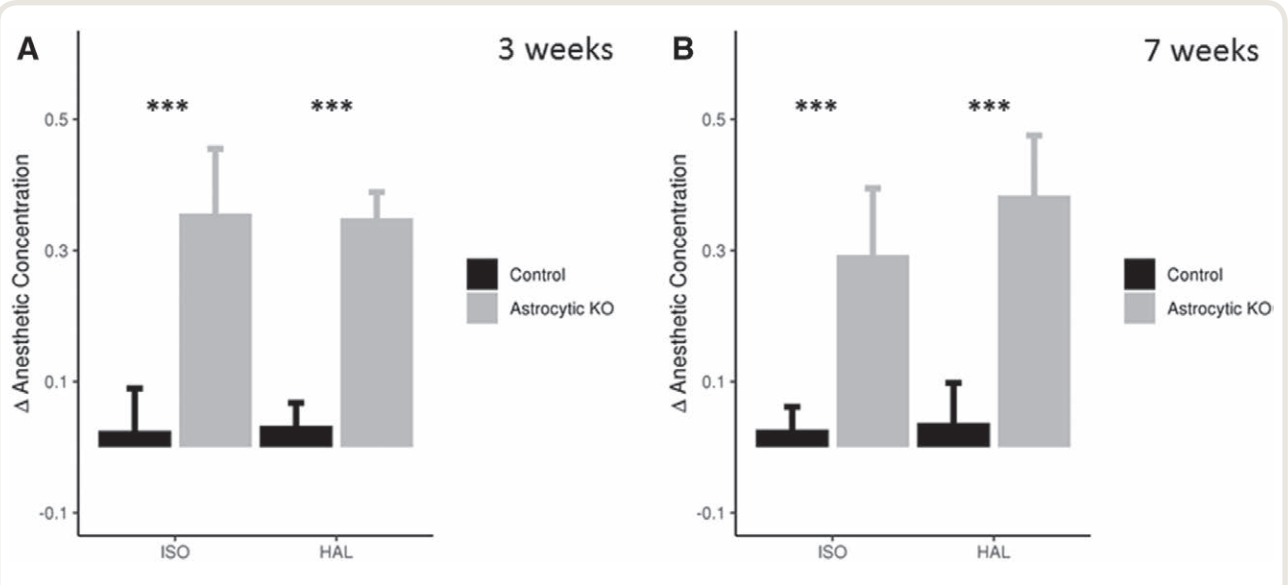

Fig. 4. Astrocyte-specific *Ndufs4(KO)* displays hysteresis in loss of righting reflex. (*A*) The change in the anesthetic concentration (Δ Anesthetic Concentration) between induction and emergence required to demonstrate righting reflex before and after exposure to isoflurane (ISO) or halothane (HAL), at 3 weeks postinjection of 4-hydroxy tamoxifen. N = 7 for control and the astrocyte-specific *Ndufs4(KO)*. (*B*) The change in the anesthetic concentration required to demonstrate righting reflex before and after exposure isoflurane or halothane at 7 weeks postinjection of 4-hydroxy tamoxifen. N = 6 for control and the astrocyte-specific *Ndufs4(KO)*. Tests for significance were done on the difference between the EC50s for induction and emergence: ***$P < 0.001$. The *error bars* represent SD. KO, knockout.

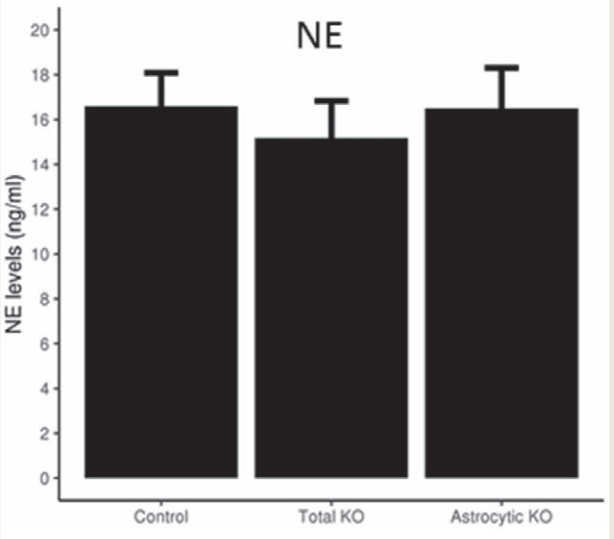

Fig. 5. Total brain norepinephrine (NE) levels are unaffected by *Ndufs4* mutation. The norepinephrine levels in the brains of mice were assessed using biochemical assay. Norepinephrine content (ng/ml) in the total brains extracts of wild-type, total *Ndufs4(KO)*, and astrocyte-specific *Ndufs4(KO)* mice. N = 6 for wild-type, total *Ndufs4(KO)*, and astrocyte-specific *Ndufs4(KO)* mice. The *error bars* represent SD. KO, knockout.

and SN2.[8,9] In addition, synaptic glutamate release, as well as neuromodulators like norepinephrine, evoke a calcium spike in astrocytes that can be transmitted between adjacent neurons and astrocytes.[17,18] This calcium signaling causes the release of neural modulators, called gliotransmitters, from astrocytes into the synapse. Gliotransmitters include adenosine triphosphate (ATP), GABA, glutamate, and D-serine, all of which modulate local neuronal function and synaptic transmission[10,19,20] at the tripartite synapse (fig. 6). It is surprising that the acute loss of NDUFS4 in astrocytes specifically lowers the concentrations at which the mutant emerges from anesthesia, without altering the induction concentrations, for both responses to tail clamp and loss of righting reflex assays. We interpret the data to indicate that the mutant mitochondria in astrocytes are inhibited by volatile anesthetics at lower concentrations than are control mitochondria. This inhibition results in decreased release of excitatory gliotransmitters and/or inhibited glutamate metabolism (fig. 6). Defective gliotransmitter release or glutamate metabolism in mutant astrocytes results in a failure of reactivation of neuronal function until volatile anesthetic concentrations are lowered sufficiently for astrocytic mitochondrial function to recover. Two important caveats must be considered: (1) we have not tested the effects of nonastrocytic nonneuronal cells, which may play a role; and (2) although we saw no evidence of compensatory changes in this acute knockout model, the existence of such changes cannot be completely ruled out.

The response to tail clamp during volatile anesthetic exposure in rodents is mediated by the spinal cord with supraspinal modification.[21,22] Previous studies have implicated the spinal cord in mediating nociception and the tail

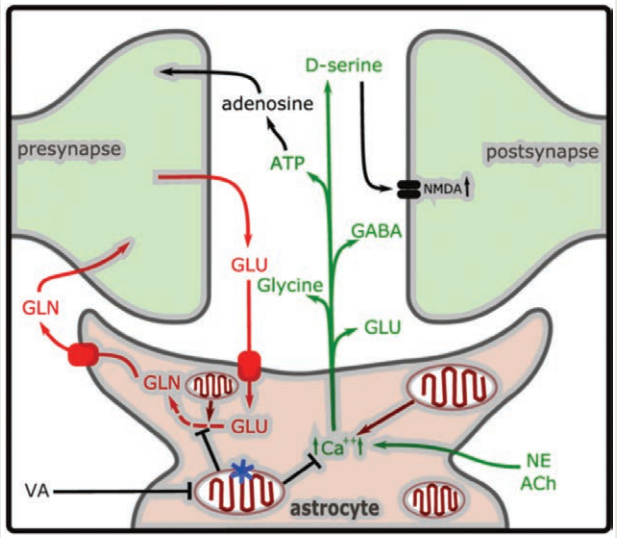

Fig. 6. A model of the tripartite synapse. Glutamate (GLU) released during synaptic transmission is taken up by GLT-1 or GLAST transporters, converted to glutamine (GLN), and released by SN1/SN2 (pathway indicated in *red*). In addition, norepinephrine (NE) or acetylcholine (ACh) stimulation during emergence causes increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), causing release of the gliotransmitters adenosine triphosphate (ATP), glutamate, D-serine, glycine, and γ-aminobutyric acid (GABA, in *green*). The Ca^{2+} homeostasis or glutamate recycling may be dysregulated in the astrocyte-specific mitochondrial mutant (indicated by a *blue asterisk*). The volatile anesthetics (isoflurane/halothane) inhibit mitochondrial complex I function at a lower concentration in *Ndufs4(KO)* mitochondria than in control mitochondria. In the case of the astrocyte-specific *Ndufs4(KO)* (mutation indicated by the *blue asterisk* in one mitochondrion), this leads to mitochondrial inhibition persisting in the astrocyte, compared to the neurons, during reduction of anesthetic concentration. We propose that this leads to neuronal inhibition from decreased excitatory gliotransmitter release or defective glutamate recycling, resulting in delayed emergence. NMDA, *N*-methyl-D-aspartate; VA, volatile anesthetics.

clamp reflex.[21,23] Although the spinal cord astrocytes have been implicated in modulating nociception,[24] their role has been mostly studied in relation to chronic neuropathic pain and allodynia. Inhibition of the astrocytic glutamate–glutamine shuttle attenuates nociceptive neuronal responsiveness in response to inflammatory and nociceptive stimuli in the medulla.[25] Alterations of these pathways caused by mitochondrial dysfunction may play a part in causing the hysteresis that we observed.

In contrast with tail clamp, the loss of righting reflex is likely mediated by interactions between the brainstem[26] and thalamocortical pathways.[27–29] These two behavioral endpoints, primarily determined by different regions of the CNS, model different human anesthetic responses (minimal alveolar concentration *vs.* loss of consciousness). Because the significant difference between induction and emergence concentrations (increased anesthetic hysteresis) was found with both of the behavioral endpoints, the emergence mechanism involved is unlikely to be dependent on one specific neuronal pathway. Instead, an astrocyte-specific mechanism is necessary for emergence with both endpoints. In addition, the structurally different anesthetics isoflurane and halothane showed that similar reductions in the concentration needed to be attained before the animals could emerge from anesthesia, suggesting that dependence of emergence on astrocytes may be a general feature of volatile anesthetic response.

Control animals and global *Ndufs4(KO)* do not display marked hysteresis presumably because both neurons and astrocytes have equal mitochondrial functions in each genotype. Mice with *Ndufs4* loss restricted to glutamatergic neurons do not display overt hysteresis either, potentially because the concentration necessary for induction is so low as to obscure the role of astrocytes in emergence. The loss of NDUFS4 solely in astrocytes reveals the critical role for this cell type in restoring consciousness.

Although the response to the tail clamp was abrupt and often strong, the return to normal activity was qualitatively different in the astrocyte-specific *Ndufs4(KO)*. Unlike the control mice, most astrocyte-specific *Ndufs4(KO)* mice displayed ataxia during the arousal period. Comparatively, the transition to the fully awake state after regaining of righting reflex was without overt ataxia, presumably because of the induction concentrations for tail clamp being higher than for loss of righting reflex. Extending our observations to the clinical setting is difficult, but our findings suggest that there could be a link between postoperative emergence trajectory and changes in glial metabolism in some individuals.

Thrane *et al.*[30] showed that anesthetic agents (ketamine/xylazine, isoflurane, urethane) depress widespread astrocytic IP3R2 (inositol 1,4,5-triphosphate type 2 receptor)–dependent Ca^{2+} transients associated with arousal in mice. One model of arousal argues that there is an evolutionarily conserved single arousal switch governing both loss and recovery of consciousness.[31–33] However, anesthesia-induced loss and recovery of consciousness have also been suggested to be two distinct states clearly defined by separate anesthetic concentration response curves.[34,35] Kelz's group[34,35] first described a difference between the concentrations needed for induction and emergence from the state of general anesthesia in *Drosophila* and in mice. In their pioneering work, they characterized this as a hysteresis, *e.g.*, arousal was in some way state-dependent. They termed this phenomenon "neural inertia."[34] Our results suggest that the neural inertia may be mediated by astrocytes and indicates that the restoration of consciousness is an energetically demanding process, inhibited by volatile anesthetics independent from the mechanisms causing initial loss of consciousness. The difference in

emergence *versus* induction concentrations of isoflurane for the astrocyte-specific *Ndufs4(KO)* was similar to that described by Kelz's group[34] for mice lacking dopamine β-hydroxylase. However, loss of righting reflex for wild-type mice in halothane was reported by Friedman *et al.*[34] to reduce the emergence/induction ratio to less than half. In the present report, control mice did not display statistically significant hysteresis under halothane or isoflurane for loss of righting reflex. Whether this disparity was due to a different stepwise decrease in halothane concentration (0.04% *vs.* our 0.1%), the manner in which loss of righting reflex was measured, the genetic background of the mouse strain, or some other technical difference is not clear.

It is important to note that astrocytes modulate both excitatory and inhibitory neuronal firing and are likely themselves regulated by metabolic status.[36–38] Astrocyte-mediated inhibition of neuronal firing can be dysregulated by astrocytic metabolic dysfunction and has been shown to result in seizure-like events in mouse cortex, counteracted by etomidate or propofol.[38] These drugs mediate their effects by accentuating GABAergic neurotransmission. Volatile anesthetics primarily mediate their effects by inhibiting excitatory neurotransmission[37,38]; our results are more consistent with astrocytic effects on excitatory synapses. The use of volatile anesthetic sensitivity as an endpoint in our studies appears to isolate the anesthetic effects on excitatory rather than inhibitory synapses. Because we previously did not see a major role for loss of NDUFS4 in GABAergic neurons in mediating anesthetic sensitivity,[39] any effects of astrocytes on inhibitory neurotransmission is unlikely to be observed with our anesthetic endpoints. In the absence of anesthetic, the suboptimal mitochondrial function in mutant astrocytes appears to be sufficient to avoid the excitatory effects seen by others with more profound defective astrocyte function.[36,38] The emergence defect of the astrocyte-specific *Ndufs4(KO)* reflects an inability of the astrocytic metabolic mutant to reinitiate excitatory firing postinduction probably because of a persistent mitochondrial inhibition in the astrocyte at relatively low volatile anesthetic concentrations.

The simplest explanation of our results is that lack of ATP in astrocytes directly alters gliotransmission, resulting in downstream inability to regain excitatory synaptic function in the presence of low concentrations of volatile anesthetic. However, whether the mitochondrial dysfunction alters ATP levels or disrupts either Ca^{2+} homeostasis or glutamate recycling is not known. Although astrocytes are classically thought to rely on aerobic glycolysis[40,41] during synaptic transmission, the astrocytic somas contain the same volume of mitochondria as neuronal cell bodies,[42] implying important mitochondrial functions in astrocytes.[43–45] During resting states, astrocytic oxidative metabolism accounts for 30% of the cortical oxygen consumption, while occupying 20 to 25% of the total volume.[46,47] The recognition that astrocytes are equivalent to neurons in their oxidative capacities signals a shift in the classical perception of astrocytes as primarily glycolytic, with broad impacts for the functional roles of the cells during resting and active states.

We also explored whether adrenergic signaling is affected by the *Ndufs4(KO)*. Norepinephrine and acetyl choline are important extracellular activating signals to cortical astrocytes[48,49] and have been associated with increasing astrocytic Ca^{2+} transients[16,50,51] and inducing the release of gliotransmitters.[19,52] Selective activation of the *locus coeruleus* neurons in rats under deep isoflurane anesthesia was shown to reduce burst suppression and shift electroencephalogram power from δ to θ, indicative of cortical arousal, and accelerate behavioral emergence.[53] We observed that the total norepinephrine content is unaltered between the global and astrocyte-specific *Ndufs4* knockouts and wild-type mice; it is still possible that the release or regional distribution of norepinephrine might be affected, rather than its production.

In summary, we have discovered an unexpected phenotype from acute loss of *Ndufs4* in astrocytes. Loss of *Ndufs4* in astrocytes did not change the volatile anesthetic concentrations required to induce anesthesia. However, the concentrations at which emergence occurred were significantly lower in the astrocyte-specific *Ndufs4(KO)* indicating a hysteresis in anesthetic sensitivity, produced by astrocytes alone.

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Competing Interests

The authors declare no competing interests.

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