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Glutamate transporter type 3 knockout mice have a decreased isoflurane requirement to induce loss of righting reflex

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Abstract

Excitatory amino acid transporters (EAAT) uptake extracellular glutamate, the major excitatory neurotransmitter in the brain. EAAT type 3 (EAAT3), the main neuronal EAAT, is expressed widely in the central nervous system. We have shown that the volatile anesthetic isoflurane increases EAAT3 activity and trafficking to the plasma membrane. Thus, we hypothesize that EAAT3 mediates isoflurane-induced anesthesia. To test this hypothesis, the potency of isoflurane to induce immobility and hypnosis, two major components of general anesthesia, was compared in the CD-1 wild-type mice and EAAT knockout mice that had a CD-1 strain gene background. Hypnosis was assessed by loss of righting reflex in this study. The expression of EAAT1 and EAAT2, two widely expressed EAATs in the central nervous system, in the cerebral cortex and spinal cord was not different between the EAAT3 knockout mice and wild-type mice. The concentration required for isoflurane to cause immobility to painful stimuli, a response involving primarily reflex loops in the spinal cord, was not changed by EAAT3 knockout. However, the EAAT3 knockout mice were more sensitive to isoflurane-induced hypnotic effects, which may be mediated by hypothalamic sleep neural circuits. Interestingly, the EAAT3 knockout mice did not have an altered sensitivity to the hypnotic effects caused by ketamine, an intravenous anesthetic that is a glutamate receptor antagonist and does not affect EAAT3 activity. These results suggest that EAAT3 modulates the sensitivity of neural circuits to isoflurane. These results, along with our previous findings that isoflurane increases EAAT3 activity, indicate that EAAT3 may regulate isoflurane-induced behavioral changes, including anesthesia.

Keywords

anesthesia; glutamate transporter; gene expression; hypnosis; isoflurane

Introduction

Glutamate transporters, also called excitatory amino acid transporters (EAATs), uptake extracellular glutamate under physiological conditions (Danbolt, 2001). This function has been considered as an important mechanism to regulate extracellular concentrations of glutamate (Clements et al., 1992; Danbolt, 2001) the major excitatory neurotransmitter in the central nervous system, because no extracellular enzyme has been identified to metabolize glutamate. Thus, EAATs have been shown to play an important role in regulating glutamate neurotransmission and preventing extracellular glutamate accumulation to levels that can cause excitotoxicity (Danbolt, 2001). Five EAATs have been identified so

far. EAAT1 and EAAT2 are glial. EAAT3 and EAAT4 are neuronal (Rothstein et al., 1994; Danbolt, 2001). EAAT5 is found in glial cells and neurons of the retina. EAAT2 and EAAT3 exist in many brain regions, including cerebral cortex and hippocampus; whereas EAAT1 is abundantly expressed in cerebellum and EAAT4 is only found in cerebellum. Quantitatively, EAAT2 and EAAT3 are the major glial and neuronal EAATs, respectively (Rothstein, et al., 1994; Lehre et al., 1995; Danbolt, 2001; Sepkuty et al., 2002; Mathews and Diamond, 2003).

EAAT3 is distributed in the perisynaptic areas and cell bodies of both glutamatergic and γ -aminobutyric acid (GABA) ergic neurons (Rothstein, et al., 1994; Danbolt, 2001). EAAT3 uptakes extracellular glutamate that can be used for the synthesis of GABA in the GABAergic neurons (Sepkuty, et al., 2002; Mathews and Diamond, 2003). Thus, EAAT3 functions may include limiting glutamate neurotransmission and enhancing the strength of GABA neurotransmission (Diamond, 2001; Sepkuty, et al., 2002; Mathews and Diamond, 2003). Increase of GABA neurotransmission and/or decrease of glutamate neurotransmission have been proposed to be the mechanisms for anesthesia (Campagna et al., 2003). We have shown that volatile anesthetics, such as isoflurane, increased EAAT3 activity (Huang and Zuo, 2005; Huang et al., 2006). We also have shown that inhibition of EAATs in the spinal cord increased the minimum alveolar concentration (MAC, the concentration at which 50% of animals do not have a motor response to painful stimuli) of isoflurane in rats (Cechova and Zuo, 2006). Thus, we hypothesize that EAAT3 is involved in isoflurane-induced anesthesia.

Materials and Methods

The animal protocol was approved by the institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80-23) revised in 1996.

Animals

The EAAT3 knockout mice were descendants of the strain established by Peghinni et al. (Peghini et al., 1997). The exon 1 of *eaat3* gene in these mice is disrupted by a neomycin resistance cassette. These mice were backcrossed with wild-type CD-1 mice for more than 10 generations to produce a strain of EAAT3 knockout mice before our study. The breeding scheme included backcrossing the EAAT3 knockout mice with wild-type CD-1 mice at least once every 8 generations to prevent genetic drift as recommended from the Banbury Conference (Silva et al., 1997). The CD-1 wild-type mice were from Charles River Laboratories (Wilmington, MA).

Western blotting

Male 8-weeks old CD-1 wild-type and EAAT3 knockout mice were euthanized by 5% isoflurane and then were immediately transcardially perfused by saline. Their brain cortices and spinal cords were collected and homogenized in lysis buffer (200 mM mannitol and 80 mM HEPES, pH 7.4) containing protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). The tissue lysates were centrifuged at 1000 g for 10 min at 4°C. The supernatant was centrifuged again at 100,000 g for 1 h at 4°C. The pellet was resuspended in lysis buffer for Western blot. The primary antibodies used were the rabbit polyclonal anti-EAAT1 antibody (1:1000 dilution; Cell Signaling Technology, Inc., MA, USA), the rabbit polyclonal anti-EAAT2 antibody (1:2000 dilution; Cell Signaling Technology, Inc.), the rabbit polyclonal anti-EAAT3 antibody (1:2000 dilution; Alpha Diagnostic International Inc., TX, USA), and the rabbit polyclonal anti-actin antibody (1:4000 dilution; Sigma-

Aldrich). The protein bands were visualized with the enhanced chemiluminescence methods. The densities of EAAT1 and EAAT2 protein bands were normalized to those of actin from the same samples to control for variations in protein sample loading and transferring during Western analysis. The results of the EAAT3 knockout mice were then normalized to those of the CD-1 wild-type mice on the same film to control for variations caused by different exposure times of films.

Loss of righting reflex determination

Twelve male EAAT3 knockout mice at age of 70 - 74 days and twelve male CD-1 wild-type mice of 64 - 70 days old were used in these experiments. As described before (Kelz et al., 2008; Bianchi et al., 2010), the mice first were habituated by staying in a gas-tight plexiglass chamber (~ 1.5 l in volume) gassed with 3 l/min of 100% oxygen for 90 min each day for two consecutive days. The chamber was partially submersed in a 37°C water bath to maintain its temperature between 36°C to 38°C. On the third day, anesthesia was induced with isoflurane (Abbott Laboratories, North Chicago, IL, USA) delivered by an agent specific vaporizer to the chamber. This was performed by stepwise increases in isoflurane concentration in oxygen. The isoflurane concentrations in the chamber were continuously monitored by a Datex infrared analyzer (Capnomac, Helsinki, Finland). The average initial isoflurane concentration was 0.59%. Isoflurane concentration was increased by an average of 0.04% for every 15 min. At the end of each 15 min interval, the chamber was rotated 180 degrees to turn the mouse upside down. If the mouse remained on its back with at least three paws up in the air for 120 s, its righting reflex was considered to be lost. At the end of the 15 min period, the entire process was repeated for the next concentration until the isoflurane concentration that induced loss of righting reflex of the mouse was identified.

The emergence time was determined in the following way. After the mouse lost its righting reflex, isoflurane concentration was increased by one more step. Isoflurane delivery then was discontinued but the chamber still was gassed with pure oxygen. Emergence time from anesthesia was the duration from stopping isoflurane delivery to recovery of the righting reflex.

The potency of ketamine to cause mice to lose righting reflex was evaluated by using a method similar to that for assessing isoflurane potency. Briefly, ketamine hydrochloride from Fort Dodge Animal Health (Fort Dodge, IA, USA) was dissolved freshly in 0.9% saline solution on the day of the experiment. It was administered intraperitoneally in a volume of 0.05 ml per 10 g of mouse body weight. The mouse then was placed in the gas-tight plexiglass chamber that was gassed with 100% oxygen and partially submerged in a 37°C water bath. The righting reflex was assessed and recorded at 2, 4, 6, 8, 10, 20, 60 and 180 min after ketamine administration. Five to six male mice of 7 - 9 weeks old were used per ketamine dose and six doses (40, 50, 60, 70, 80 and 100 mg/kg) were tested to construct a dose-response curve as described before (Irifune et al., 2007).

MAC measurement

Eight male CD-1 wild-type mice and eight male EAAT3 knockout mice at age of 7 to 9 weeks were used in the study. A mouse was placed in a temperature-controlled chamber as described above for determining the isoflurane concentrations required to induce loss of righting reflex. The chamber has two holes; one is for delivering oxygen with anesthetics and the other for the mouse tail to pass through. The mouse was not restrained other than its tail was held in place by a piece of tape after it had passed through the chamber hole. MAC was measured in a similar way as we described before (Cechova and Zuo, 2006). Briefly, the initial isoflurane concentration was 0.8%. An 8 inch hemostat was clamped to the first ratchet lock on the tail for 1 min. The next stimulation site on the tail is always proximal to

the previous test site. If an animal responded to the tail clamp with gross movement of the head, extremities or body, the isoflurane concentration was increased in steps of approximately 0.1% until no response was obtained. The animals breathed isoflurane at a particular concentration for 30 min to achieve tissue equilibration before the test of their responses. MAC was estimated to be the concentration midway between the highest concentration permitting a positive test (responding to pain with gross movement) and the lowest concentration having a negative test.

Statistical analysis

Data are presented as mean $\pm$ S.D. The results of Western blotting between the CD-1 wild-type mice and EAAT3 knockout mice were analyzed by Student's t-test. This analysis was performed with SigmaStat software (SYSTAT Software, Inc., Point Richmond, CA, USA). Dose-response results were analyzed and plotted by using Prism 4.0 (GraphPad Software, San Diego, CA, USA). The mean EC₅₀, ED₅₀ and Hill coefficients and their corresponding 95% confidence intervals were calculated. A Fisher's exact test was used to determine whether results from the best-fit curves were different between the CD-1 wild-type and EAAT3 knockout mice. A $P < 0.05$ was accepted as significant.

Results

The EAAT3 knockout mice did not express EAAT3 proteins in their brains and spinal cords (Fig. 1). These mice expressed EAAT1 and EAAT2 proteins in the brain and spinal cord at a level similar to that of CD-1 wild-type mice (Fig. 1).

The EC₅₀ for isoflurane to induce loss of righting reflex of the CD-1 wild-type mice was 0.82% (Fig. 2 and table 1). This EC₅₀ is similar to that reported previously for mice (Kelz, et al., 2008; Bianchi, et al., 2010). The dose-response curve for the EAAT3 knockout mice was left-shifted with a significantly lower EC₅₀ (0.75%). However, there was no difference in the Hill coefficients between the wild-type and EAAT3 knockout mice (Table 1). The emergency time from anesthesia was also not different between these two groups of mice (Fig. 2).

The EC₅₀ for isoflurane to prevent motor responses to painful stimuli, a concept that is usually called MAC, was 1.31% in the CD-1 wild-type mice (Fig. 3 and Table 1). This MAC value is similar to that reported previously for mice (Joo et al., 2001). The dose-response curves of the wild-type and EAAT3 knockout mice overlapped very well. Thus, there were no differences in the EC₅₀ or Hill coefficients for isoflurane to prevent motor response to painful stimuli in the wild-type and EAAT3 knockout mice (Fig. 3 and Table 1).

The ED₅₀ for ketamine to induce loss of righting reflex in the wild-type mice was 61 mg/kg (Fig. 4 and Table 2), which is similar to that reported for mice in the literature (Irifune, et al., 2007). As shown in figure 4 and table 2, the ED₅₀ and Hill coefficients for ketamine to induce loss of righting reflex were not different between the CD-1 wild-type and EAAT3 knockout mice.

Discussion

EAAT3, the major neuronal EAAT, has been proposed to regulate both glutamatergic neurotransmission and GABAergic neurotransmission (Sepkuty, et al., 2002; Mathews and Diamond, 2003). A simple view of anesthesia mechanism is that anesthesia is induced by enhancing the inhibitory neurotransmission and/or decreasing the excitatory neurotransmission (Campagna, et al., 2003). Glutamate and GABA are the major excitatory

and inhibitory neurotransmitters, respectively, in the brain. Thus, it is possible that EAAT3 may regulate anesthetic requirement to induce anesthesia.

General anesthesia includes at least three components: hypnosis, immobility and amnesia (Campagna, et al., 2003; Sonner et al., 2003). The potency of anesthetics to induce immobility is often measured by MAC values. We have shown in a previous study that intrathecal injection of a general EAAT inhibitor or a relatively specific EAAT2 inhibitor increases the MAC for isoflurane in rats (Cechova and Zuo, 2006), suggesting that EAATs, especially EAAT2, may be involved in regulating isoflurane requirement to induce immobility. The involvement of EAAT1 and EAAT3 in anesthetic-induced immobility has not been investigated due to the lack of specific inhibitors for them. Here, we show that the wild-type and EAAT3 knockout mice have a similar MAC for isoflurane, suggesting that EAAT3 may not be involved in isoflurane-induced immobility.

Loss of righting reflex is often used to evaluate anesthetic potency to induce hypnosis in animal studies (Kelz, et al., 2008; Bianchi, et al., 2010). The involvement of EAATs in anesthetic-induced hypnosis has not been studied. We show here that the EAAT3 knockout mice required significantly lower concentrations of isoflurane to lose their righting reflexes, suggesting that these mice are more sensitive than the wild-type mice to isoflurane-induced hypnosis. These results also suggest that EAAT3 is responsible for the altered requirement for isoflurane to induce hypnosis. This suggestion is supported by the finding that the potency of ketamine to induce loss of righting reflex of the EAAT3 knockout mice was not different from that in the wild-type mice in our study. Ketamine is a glutamate receptor blocker (Anis et al., 1983) that does not affect EAAT3 activity (Yun et al., 2006). These findings, along with our previous results showing that isoflurane increases EAAT3 activity and trafficking to the plasma membrane (Huang and Zuo, 2005; Huang, et al., 2006), indicate that EAAT3 is a molecular target for the effects of isoflurane in the brain.

The findings that EAAT3 knockout mice had an increased sensitivity to isoflurane-induced hypnosis are seemingly contradictory to what one will predict based on EAAT3's functions. EAAT3 uptakes extracellular glutamate that can be used as a substrate to synthesize GABA in neurons. Loss of EAAT3 function may prolong glutamate neurotransmission and decrease the strength of GABA neurotransmission and, therefore, should increase the requirement of isoflurane to induce hypnosis. The reasons for the discrepancy between our results and predicted results are not known. Multiple mechanisms can be proposed to explain this discrepancy. For example, loss of EAAT3 in the inhibitory GABAergic interneurons may increase the activity of their downstream inhibitory neurons, which then may decrease the requirement of isoflurane to induce anesthesia. Also, loss of EAAT3 can prolong the stay of glutamate in the extracellular space. This effect may desensitize glutamate receptors, which can increase the sensitivity of neurons to isoflurane for inhibition.

It has been convincingly shown that the spinal cord is the primary site for mediating immobility (Antognini and Schwartz, 1993). Multiple brain regions including hypothalamic nuclei and cerebral cortex may be involved in the anesthetic-induced hypnosis (Kelz, et al., 2008). These brain regions and spinal cord express EAAT3 (Rothstein, et al., 1994; Danbolt, 2001). However, the EAAT3 knockout mice had an isoflurane MAC value similar to that of the wild-type mice and required a lower concentration of isoflurane to induce hypnosis than wild-type mice. These different responses of EAAT3 knockout mice in the tests measuring immobility and hypnosis suggest that different anatomic sites may be involved in general anesthetic-induced immobility and hypnosis, a phenomenon that is well-established in the literature (Antognini and Schwartz, 1993; Campagna, et al., 2003). These different responses may also indicate that neural circuits with different complexities mediate the anesthetic-induced immobility and hypnosis (Antognini and Schwartz, 1993; Campagna, et

al., 2003; Kelz, et al., 2008; Zecharia et al., 2009). A possibly more complex neural circuit involving many interneurons for hypnosis may have amplified the effects of EAAT3, which may not be evident in a simple spinal cord reflex loop that mediates general anesthetic-induced immobility.

The EAAT3 knockout mice and wild-type littermates have similar phenotypes for fertility, litter size, growth, brain structure, learning, memory and sensitivity to the convulsant pentamethylenetetrazole (Peghini, et al., 1997). Although the EAAT3 knockout mice appear normal at the age used in our study, one of the major problems with using genetic modified animals is compensatory expression changes of proteins related to the gene that is modified. We showed here that the expression of EAAT1 and EAAT2 proteins in the spinal cord and cerebral cortex of the EAAT3 knockout mice was not changed, suggesting that compensatory changes in EAAT1 and EAAT2 expression does not occur in the EAAT3 knockout mice. It is not known yet whether many other components involved in glutamate neurotransmission, such as glutamate receptors, have a compensatory change in the EAAT3 knockout mice. Very significant changes of glutamate receptors in the EAAT3 knockout mice may not be likely because the dose-response curves for ketamine to induce hypnosis in the wild-type and EAAT3 knockout mice overlap. Altered sensitivity of the EAAT3 knockout mice to the hypnotic effects of isoflurane may be due to changed pharmacokinetics in these mice. However, this possibility is very unlikely because EAAT3 knockout mice had a similar MAC value and emergency time from anesthesia to those of the wild-type mice.

Among the five EAATs, EAAT2 and EAAT3 have a wider distribution than other EAATs in the adult rodent central nervous system and are considered to be the major glial and neuronal EAATs, respectively (Rothstein, et al., 1994; Lehre, et al., 1995; Danbolt, 2001; Sepkuty, et al., 2002; Mathews and Diamond, 2003). EAAT2 is in the glial plasma membrane surrounding synapses and is estimated to account for more than 90% of the total glutamate transport capacity in the brain (Maragakis and Rothstein, 2004). Down-regulation of EAAT2 causes an increased extracellular glutamate concentration and neurodegeneration characteristic of excitotoxicity (Rothstein et al., 1996). EAAT2 knockout mice have a significant hippocampal neuronal loss, epileptic activity and 50% mortality at 6 weeks of age (Tanaka et al., 1997). Thus, it has been proposed that EAAT2 plays a major role in maintaining normal extracellular glutamate concentrations and regulating glutamate neurotransmission. EAAT3 is expressed in the perisynaptic membrane of the postsynaptic neurons (Danbolt, 2001). It can transport cysteine into neuron very effectively for the synthesis of glutathione (Aoyama, et al., 2006). Recent results suggest that EAAT3 can buffer glutamate released from synapses to limit spillover between synapses and facilitate long-term potentiation (Scimemi et al., 2009). In addition, EAAT3 redistributes to the plasma membrane of the hippocampal neurons during learning and memory processes in rodents (Levenson et al., 2002). These results suggest that EAAT3 is involved in learning and memory functions. Our current results indicate an involvement of EAAT3 in isoflurane-induced hypnosis, another function for EAAT3 in rodent brains.

In summary, we found that the EAAT3 knockout mice had an increased sensitivity to isoflurane-induced hypnosis and did not change the requirement for isoflurane to induce immobility or ketamine to cause hypnosis. These results indicate that EAAT3 regulates the sensitivity of neural circuits to important drugs, such as general anesthetics. The direction of altered sensitivity may be difficult to predict purely based on protein functions in a single neuron model. These results, along with our findings that isoflurane increases EAAT3 activity and trafficking to the plasma membrane, suggest that EAAT3 may be a mediator for the effects of volatile anesthetics on the central nervous system.

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Abbreviations

EAAT	excitatory amino acid transporters
GABA	γ -aminobutyric acid
MAC	minimum alveolar concentration

References

- Anis NA, Berry SC, Burton NR, Lodge D. The dissociative anaesthetics, ketamine and phencyclidine, selectively reduce excitation of central mammalian neurones by N-methyl-aspartate. *Br J Pharmacol.* 1983; 79:565–575. [PubMed: 6317114]
- Antognini JF, Schwartz K. Exaggerated anesthetic requirements in the preferentially anesthetized brain. *Anesthesiology.* 1993; 79:1244–1249. [PubMed: 8267200]
- Aoyama K, Suh SW, Hamby AM, Liu J, Chan WY, Chen Y, Swanson RA. Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. *Nat Neurosci.* 2006; 9:119–126. [PubMed: 16311588]
- Bianchi SL, Caltagarone BM, Laferla FM, Eckenhooff RG, Kelz MB. Inhaled anesthetic potency in aged Alzheimer mice. *Anesth Analg.* 2010; 110:427–430. [PubMed: 19820240]
- Campagna J, Miller K, Forman S. Mechanisms of action of inhaled anesthetics. *N Engl J Med.* 2003; 348:2110–2124. [PubMed: 12761368]
- Cechova S, Zuo Z. Inhibition of glutamate transporters increases the minimum alveolar concentration for isoflurane in rats. *Br J Anaesth.* 2006; 97:192–195. [PubMed: 16793777]
- Clements JD, Lester RA, Tong G, Jahr CE, Westbrook GL. The time course of glutamate in the synaptic cleft. *Science.* 1992; 258:1498–1501. [PubMed: 1359647]
- Danbolt NC. Glutamate uptake. *Prog Neurobiol.* 2001; 65:1–105. [PubMed: 11369436]
- Diamond JS. Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. *J Neurosci.* 2001; 21:8328–8338. [PubMed: 11606620]
- Huang Y, Zuo Z. Isoflurane induces a protein kinase C α -dependent increase in cell surface protein level and activity of glutamate transporter type 3. *Mol Pharmacol.* 2005; 67:1522–1533. [PubMed: 15709112]
- Huang Y, Feng X, Sando JJ, Zuo Z. Critical Role of Serine 465 in Isoflurane-induced Increase of Cell-surface Redistribution and Activity of Glutamate Transporter Type 3. *J Biol Chem.* 2006; 281:38133–38138. [PubMed: 17062570]
- Irifune M, Katayama S, Takarada T, Shimizu Y, Endo C, Takata T, Morita K, Dohi T, Sato T, Kawahara M. MK-801 enhances gabaculine-induced loss of the righting reflex in mice, but not immobility. *Can J Anaesth.* 2007; 54:998–1005. [PubMed: 18056209]
- Joo DT, Gong D, Sonner JM, Jia Z, MacDonald JF, Eger EI 2nd, Orser BA. Blockade of AMPA receptors and volatile anesthetics: reduced anesthetic requirements in GluR2 null mutant mice for loss of the righting reflex and antinociception but not minimum alveolar concentration. *Anesthesiology.* 2001; 94:478–488. [PubMed: 11374610]
- Kelz MB, Sun Y, Chen J, Cheng Meng Q, Moore JT, Veasey SC, Dixon S, Thornton M, Funato H, Yanagisawa M. An essential role for orexins in emergence from general anesthesia. *Proc Natl Acad Sci U S A.* 2008; 105:1309–1314. [PubMed: 18195361]

- Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. *J Neurosci.* 1995; 15:1835–1853. [PubMed: 7891138]
- Levenson J, Weeber E, Selcher JC, Kategaya LS, Sweatt JD, Eskin A. Long-term potentiation and contextual fear conditioning increase neuronal glutamate uptake. *Nat Neurosci.* 2002; 5:155–161. [PubMed: 11788834]
- Maragakis NJ, Rothstein JD. Glutamate transporters: animal models to neurologic disease. *Neurobiol Dis.* 2004; 15:461–473. [PubMed: 15056453]
- Mathews GC, Diamond JS. Neuronal glutamate uptake contributes to GABA synthesis and inhibitory synaptic strength. *The Journal of Neuroscience.* 2003; 23:2040–2048. [PubMed: 12657662]
- Peghini P, Janzen J, Stoffel W. Glutamate transporter EAAC-1-deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. *EMBO J.* 1997; 16:3822–3832. [PubMed: 9233792]
- Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW. Localization of neuronal and glial glutamate transporters. *Neuron.* 1994; 13:713–725. [PubMed: 7917301]
- Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron.* 1996; 16:675–686. [PubMed: 8785064]
- Scimemi A, Tian H, Diamond JS. Neuronal transporters regulate glutamate clearance, NMDA receptor activation, and synaptic plasticity in the hippocampus. *J Neurosci.* 2009; 29:14581–14595. [PubMed: 19923291]
- Sepkuty JP, Cohen AS, Eccles C, Rafiq A, Behar K, Ganel R, Coulter DA, Rothstein JD. A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy. *The Journal of Neuroscience.* 2002; 22:6372–6379. [PubMed: 12151515]
- Silva AJ, Simpson EM, Takahashi JS, Lipp H-P, Nakanishi S, Wehner JM, Giese KP, Tully T, Abel T, Chapman PF, Fox K, Grant S, Itoharu S, Lathe R, Mayford M, McNamara JO, Morris RJ, Picciotto M, John Roder, Hee-Sup Shin, et al. Mutant mice and neuroscience: recommendations concerning genetic background. *Banbury Conference on genetic background in mice. Neuron.* 1997; 19:755–759. [PubMed: 9354323]
- Sonner JM, Antognini JF, Dutton RC, Flood P, Gray AT, Harris RA, Homanics GE, Kendig J, Orser B, Raines DE, Rampil IJ, Trudell J, Vissel B, Eger EI 2nd. Inhaled anesthetics and immobility: mechanisms, mysteries, and minimum alveolar anesthetic concentration. *Anesth Analg.* 2003; 97:718–740. [PubMed: 12933393]
- Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science.* 1997; 276:1699–1702. [PubMed: 9180080]
- Yun JY, Kim JH, Kim HK, Lim YJ, Do SH, Zuo Z. Effects of intravenous anesthetics on the activity of glutamate transporter EAAT3 expressed in *Xenopus* oocytes: evidence for protein kinase C involvement. *Eur J Pharmacol.* 2006; 531:133–139. [PubMed: 16413532]
- Zecharia AY, Nelson LE, Gent TC, Schumacher M, Jurd R, Rudolph U, Brickley SG, Maze M, Franks NP. The involvement of hypothalamic sleep pathways in general anesthesia: testing the hypothesis using the GABAA receptor beta3N265M knock-in mouse. *J Neurosci.* 2009; 29:2177–2187. [PubMed: 19228970]

Fig. 1. Glutamate transporter (EAAT) expression

Cerebral cortex (panel A) and spinal cord (panel B) of the CD-1 wild-type (WT) mice and EAAT3 knockout ($-/-$) mice were harvested for Western blotting. A representative Western blot is shown on the top panel and the graphic presentation of the EAAT protein abundance quantified by integrating the volumes of autoradiograms from 8 CD-1 wild-type mice and 8 EAAT3 knockout mice is shown in the bottom panel. Values in graphs are expressed as fold change over the mean values of the CD-1 wild-type mice and presented as the means $\pm$ S.D.

Fig. 2. Effects of isoflurane on righting reflex

Panel A: Dose-response curves of isoflurane-induced loss of righting reflex (n = 12 to generate each data point); Panel B: Average concentrations of isoflurane to induce loss of righting reflex; and Panel C: Emergence time from anesthesia. Results in panel B and panel C are presented as the means $\pm$ S.D. (n = 12). * P < 0.01 compared with the CD-1 wild-type mice. EAAT3^{-/-}: glutamate transporter type 3 knockout mice.

Fig. 3. Effects of isoflurane on motor response to painful stimuli

Panel A: Dose-response curves of isoflurane-induced loss of motor response to painful stimuli ($n = 8$ to generate each data point); and Panel B: Average concentrations of isoflurane to prevent motor response to painful stimuli. Results in panel B are presented as the means $\pm$ S.D ($n = 8$). EAAT3^{-/-}: glutamate transporter type 3 knockout mice.

Fig. 4. Effects of ketamine on righting reflex

Dose-response curves of ketamine-induced loss of righting reflex are shown ($n = 5 - 6$ to generate each data point). EAAT3^{-/-}: glutamate transporter type 3 knockout mice.

Table 1

Concentration-response parameters of isoflurane-induced hypnosis and immobility

	<u>Loss of righting reflex</u>		<u>Loss of response to painful stimuli</u>	
	EC50 (% atm)	Hill slope	EC50 (% atm)	Hill slope
Wild-type	0.82 (0.82–0.83)	37 (30–45)	1.31 (1.27–1.35)	13 (8–19)
EAAT3 ^{-/-}	0.75 (0.74 – 0.75)	42 (34 – 50)	1.28 (1.23–1.32)	14 (8–21)

Results are mean (95% confidence interval). EAAT3^{-/-} glutamate transporter type 3 knockout mice; EC50: 50% effective concentration; atm: atmosphere.

Table 2

Dose-response parameters of ketamine-induced loss of righting reflex

	ED50 (mg/kg)	Hill slope
Wild-type	61 (56–67)	11.2 (0.4–22.0)
EAAT3 ^{-/-}	70 (57 – 85)	5.8 (0.5–11.1)

Results are mean (95% confidence interval). EAAT3^{-/-}: glutamate transporter type 3 knockout mice; ED50: 50% effective dose.