Mutation of α_{1G} T-type Calcium Channels in Mice Does Not Change Anesthetic Requirements for Loss of the Righting Reflex and Minimum Alveolar Concentration but Delays the Onset of Anesthetic Induction

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Background: T-type calcium channels regulate neuronal membrane excitability and participate in a number of physiologic and pathologic processes in the central nervous system, including sleep and epileptic activity. Volatile anesthetics inhibit native and recombinant T-type calcium channels at concentrations comparable to those required to produce anesthesia. To determine whether T-type calcium channels are involved in the mechanisms of anesthetic action, the authors examined the effects of general anesthetics in mutant mice lacking α_{1G} T-type calcium channels.

Methods: The hypnotic effects of volatile and intravenous anesthetics administered to mutant and C57BL/6 control mice were evaluated using the behavioral endpoint of loss of righting reflex. To investigate the immobilizing effects of volatile anesthetics in mice, the minimum alveolar concentration (MAC) values were determined using the tail-clamp method.

Results: The 50% effective concentration for loss of righting reflex and MAC values for volatile anesthetics were not altered after α_{1G} channel knockout. However, mutant mice required significantly more time to develop anesthesia/hypnosis after exposure to isoflurane, halothane, and sevoflurane and after intraperitoneal administration of pentobarbital.

Conclusions: The 50% effective concentration for loss of righting reflex and MAC values for the volatile anesthetics were not altered after α_{1G} calcium channel knockout, indicating that normal functioning of α_{1G} calcium channels is not required for the maintenance of anesthetic hypnosis and immobility. However, the timely induction of anesthesia/hypnosis by volatile anesthetic agents and some intravenous anesthetic agents may require the normal functioning of these channel subunits.

CALCIUM channels have been classified pharmacologically and biophysically into six distinct types designated as L, T, N, P, Q, and R types.[1] Low-voltage–activated T-type channels are distinguished from high-voltage–activated L-, N-, P-, Q-, and R-type channels based on the relatively hyperpolarized potential at which they are activated. Each channel type has been cloned and is composed of a large α_1 subunit that determines the type of calcium channel in combination with smaller accessory subunits. Three genes encoding α_1 subunits of T-type calcium channels, named α_{1G}, α_{1H}, and α_{1I}, have been cloned.[2] Among these, the α_{1G} transcripts are widely and predominantly expressed in many brain areas, including the cerebral cortex, thalamocortical relay nuclei, amygdala, cerebellum, brainstem, and spinal cord.[3]

One of the best-known functions of T-type channels is mediation of neuronal rebound burst firing after administration of endogenous or exogenous inhibitory postsynaptic potentials.[4] T-type channels are hypothesized to underlie neuronal pacemaker activity, oscillations in neuronal membrane potential, input signal amplification, and synaptic integration in neuronal networks.[4] T-type channels are also known to control the rate of action potentials in the thalamus,[5] which serves as a gateway to the cerebral cortex and is suggested to be important for the mechanisms of physiologic sleep and anesthetic-induced unconsciousness.[6]

Volatile anesthetics, such as isoflurane and halothane, are reported to block T-type currents in dorsal root ganglia and hippocampal and thalamic relay neurons, as well as those due to recombinant α_{1G} T-type channels, at clinically relevant concentrations.[7–11] Therefore, the direct effects of volatile anesthetics on T-type calcium channel variants might contribute to the clinical effects of these agents. Because intravenous anesthetics such as barbiturates generally block T-type calcium channels at concentrations higher than those achieved during clinical use,[11] T-type calcium channels are unlikely to represent their anesthetic site of action. However, considering the important role of T-type calcium channels in regulating neuronal excitation, we cannot exclude the possibility of their indirect (modulatory) involvement in producing intravenous anesthesia.

To determine whether T-type calcium channels have a role in general anesthesia, we deleted the gene encoding the α_{1G} subunit and used the behavioral endpoints of loss of righting reflex (LORR) and immobility to tail clamp (minimum alveolar concentration [MAC]) to test the hypothesis that α_{1G} T-type calcium channel knockout mice would demonstrate altered sensitivity to general anesthetics.

Materials and Methods

This study was approved by the Committee for the Guidelines on Animal Experimentation of Niigata Univer-

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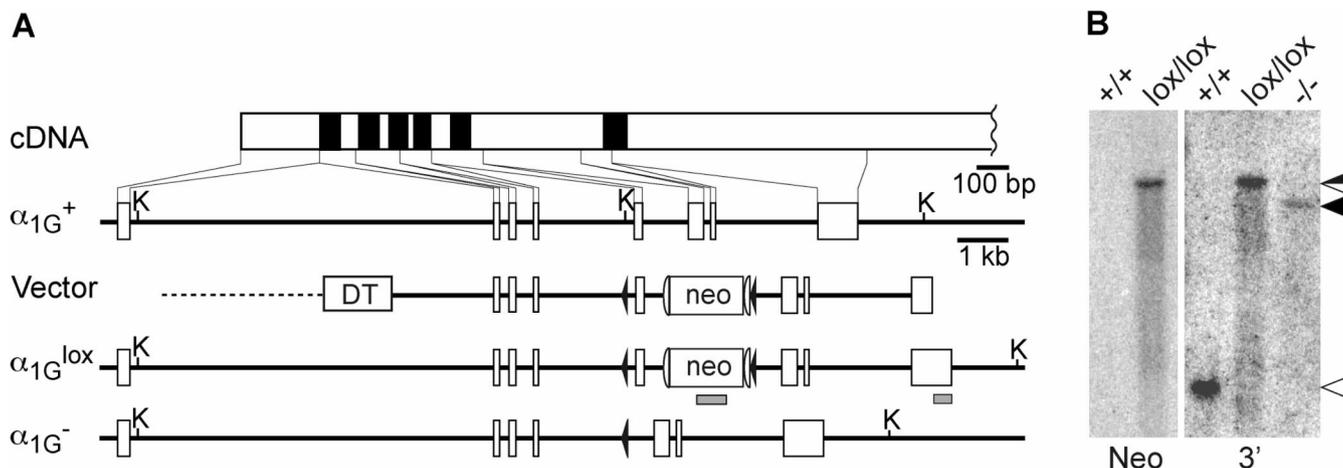

Fig. 1. Generation of α_{1G} T-type calcium channel knockout mice by a Cre/loxP recombination system. (*A*) Schematic representation of α_{1G} T-type calcium channel complementary DNA (cDNA), α_{1G}^+ allele (α_{1G}^+), targeting vector (Vector), targeted α_{1G}^{lox} allele (α_{1G}^{lox}), and α_{1G}^- allele (α_{1G}^-) after Cre-mediated recombination. *Black areas* of cDNA represent the nucleotide sequences encoding the membrane spanning segments S1–S6 of repeat I of the α_{1G} channel. *White boxes* represent exon sequences, Pgk-1 promoter-neo-pA cassette (neo), and diphtheria toxin cassette (DT). LoxP and frt sites are shown by *black triangles* and *white half-circles*, respectively. *Gray boxes* indicate probe regions (neo and 3′) used for Southern blot analysis. K is the *Kpn*I site. (*B*) Southern blot analysis of genomic DNA from $\alpha_{1G}^{+/+}$, $\alpha_{1G}^{lox/lox}$, and $\alpha_{1G}^{-/-}$ mice. *Left*: *Kpn*I-digested DNA hybridized with a neo probe. *Right*: *Kpn*I-digested DNA hybridized with a 3′ probe. The *black triangle* indicates the 14.7-kb fragment from the α_{1G}^- allele. The *white triangle* indicates the 5.9-kb fragment from the α_{1G}^+ allele. The *triangle with a black upper half* indicates the 17.2-kb fragment from the α_{1G}^{lox} allele.

sity (Niigata, Japan). All procedures were performed on male adult (aged 8–14 weeks) C57BL/6 and α_{1G} T-type calcium channel knockout mice, hereafter referred to as wild-type and mutant, respectively. Animals were housed two to four per cage under a standard 12-h light–dark cycle; water and food were available *ad libitum*. The temperature of the testing room was kept at 24°C, and experiments were conducted between 09:00 and 17:00.

Generation of α_{1G} Knockout Mice

Mutant mice were produced by homologous recombination using an embryonic stem cell line Bruce4, which originated from C57BL/6 embryos, gifted by Colin L. Stewart, Ph.D. (Laboratory Chief, Cancer and Developmental Biology Laboratory, National Cancer Institute, Frederick, Maryland). The mouse α_{1G} T-type calcium channel complementary DNA (cDNA) fragment[12] was amplified using the reverse-transcriptase polymerase chain reaction. Total RNA from C57BL/6 mouse brain was extracted using TRIzol (GIBCO BRL, Grand Island, NY) according to the manufacturer's instructions. The reverse-transcriptase polymerase chain reaction was performed according to the manufacturer's instructions (Takara Biochemicals Inc., Shiga, Japan). The following primers were used: Ca5-1, 5′-ACCCCAGCTGGCCAGAG-GATGGATGAG-3′; Ca3-4, 5′-ATAGCAGCTGCCTGGCT-CAGAGAAG-3′. The amplified fragment was subcloned and sequenced. The mouse α_{1G} T-type calcium channel gene was cloned from a C57BL/6 mouse genomic library (Clontech, Palo Alto, CA) by its hybridization with a mouse α_{1G} T-type calcium channel cDNA probe. A 15-kb genomic fragment of the α_{1G} T-type calcium channel

gene was subcloned and characterized by restriction mapping, nucleotide sequencing, and Southern blot analysis. A 1.8-kb DNA fragment carrying the 34-bp loxP sequence was inserted into a site 170 bp upstream of exon 5. A Pgk-1 promoter-driven neomycin phosphotransferase gene, flanked by two Flp recognition target sites,[13] and a second 34-bp loxP sequence were inserted into the site 330 bp downstream of exon 5. The targeting vector ptv_α_{1G}-flox (fig. 1A) contained exon 5 of the α_{1G} gene flanked by loxP sequences, 4.5 kb of genomic sequence upstream of exon 5 and a further 3.6 kb downstream of exon 5, and 4.3 kb of the vector pMC1DTpA.[14]

Embryonic stem cells were cultured on mitomycin C-treated neomycin-resistant fibroblasts in Dulbecco's modified Eagle's medium (high glucose; Invitrogen, Carlsbad, CA) supplemented with 17.7% embryonic stem–cell qualified fetal calf serum (Invitrogen), 88 μM nonessential amino acids (Invitrogen), 884 μM sodium pyruvate (Sigma, St. Louis, MO), 88 μM 2-mercaptoethanol (Sigma), and 884 U/ml murine leukemia inhibitory factor, embryonic EMGRO (Chemicon International, Temecula, CA). *Kpn*I-linearized ptv_α_{1G}-flox was electroporated into Bruce4 cells, and G-418 (175 μg/ml; Invitrogen)–resistant clones were picked. Recombinant clones were identified by Southern blot hybridization analysis. Recombinant embryonic stem cells were injected into eight-cell stage embryos of the CD-1 mouse strain. The embryos were cultured to blastocysts and transferred to pseudopregnant CD-1 mouse uteri. The resulting chimeric mice were mated to C57BL/6 mice, and offspring ($\alpha_{1G}^{+/lox}$) were further crossed with TLCN-Cre mice[15,16] to yield heterozygous ($\alpha_{1G}^{\pm}$) mice with a genetic background of C57BL/6 (99.9937%) and CBA (0.0063%). Ho-

mozygous $\alpha_{1G}^{-/-}$ mice were obtained by crossing heterozygous pairs (fig. 1B).

Mutant mice were normal in appearance, weight, and overt behavior. They demonstrated a brisk righting reflex and showed no differences in gross motor abilities under control conditions.

Behavioral Studies of Sensitivity to Volatile Anesthetics

Loss of Righting Reflex (Hypnosis) and Loss of Movement to Tail Clamp (Immobilization). Mice were individually placed in small plastic chambers connected to a vaporizer and oxygen source. Measurements were performed on two wild-type and two mutant mice in each session. Isoflurane (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), halothane (Takeda Pharmaceutical Co., Ltd., Osaka, Japan), and sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) were vaporized in 2 l/min oxygen. The concentrations of anesthetics were continually monitored using an infrared gas analyzer (Capnomac Ultima, Instrumentarium Corp., Helsinki, Finland). Each concentration of anesthetic was maintained for a minimum equilibration period of 20 min. Rectal temperatures were determined with a digital thermometer (TD-300; Shibaura Electronics, Saitama, Japan) before and after each measurement, and body temperature was actively maintained between 36° and 38°C by warming with a plastic plate filled with circulating hot water placed below the chambers.

To investigate the hypnotic properties of volatile anesthetics in mice, the behavioral endpoint of LORR was used. Isoflurane, halothane, or sevoflurane was administered to each chamber at initial concentrations of 0.9, 1.0, and 1.8% atm, respectively. After the equilibration period, the chamber was gently rotated to place the mouse on its back, and the righting reflex was monitored for up to 15 s. All mice experienced LORR at these concentrations. The concentration of each anesthetic was decreased by 0.1% atm for another equilibration period and the response was tested again. The 50% concentration for LORR (LORR ED_{50}) was calculated by averaging the two concentrations at which the mouse either retained or lost the righting reflex.

To investigate the immobilizing properties of volatile anesthetics in mice, the MAC values were determined using the classic tail-clamp paradigm. Isoflurane, halothane, or sevoflurane was administered to each chamber at initial concentrations of 0.9, 0.9, and 2.5% atm, respectively. After the equilibration period, movement to tail clamp was tested by the placement of an alligator clip on the proximal one third of the tail. All mice experienced movement to tail clamp at these concentrations. The concentration of each volatile anesthetic was increased by 0.1% atm for another equilibration period, and the response was tested again. If no movement was observed, the clamp was left in place for a 1-min period.

The concentration at which the mouse lost its tail-clamp reflex was noted. The MAC was calculated by averaging the two concentrations at which the mouse either retained or lost the movement response to tail clamp.

LORR ED_{50} and MAC were measured in the same mice. Each mouse was tested with only two anesthetics in a random order. At least 1 week was allowed between each treatment for the mice to recover.

Time to Loss of Righting Reflex and Time to Loss of Withdrawal Reflex to Tail Clamp Placement. To measure the time required to reach two specific components of the anesthetized state, hypnosis and immobility, mice were individually placed in a small plastic chamber (14 × 10 × 5 cm) with preset concentrations of volatile anesthetics vaporized in 1 l/min oxygen. The concentrations of anesthetics were monitored as described above. Mice were introduced to the chamber through a "hatch" covered with a silicone membrane cut in the form of an X, which was immediately hermetically sealed off by a plastic cap. This allowed the anesthetic concentrations to be kept constant throughout the experiment. Another opening, connected to a "sleeve" made from the finger of a rubber glove, was used to introduce two manipulators into the chamber: one with a J-shaped end for lifting the mouse tail, and another one for placing an alligator clip on the tail.

To measure the time to loss of righting reflex (TTLORR), mice were exposed to 0.8 and 1 MAC concentrations of isoflurane, halothane, or sevoflurane, determined at the beginning of the study, and the ability of mice to right themselves within 15 s after being placed on their backs by tilting the chamber was evaluated at 30-s intervals after commencing exposure. The time at which a mouse first demonstrated an LORR, confirmed by two subsequent trials, was considered the TTLORR.

To measure the time to loss of withdrawal reflex (TTLOWR), mice were exposed to 1.3 MAC concentrations of isoflurane, halothane, or sevoflurane, determined at the beginning of the study, and the response to tail clamp placement was evaluated at 30-s intervals after commencing exposure. If no movement was observed, the clamp was left in place for 30 s. The time at which a mouse first lost its tail-clamp reflex, confirmed by two subsequent clamp placements on slightly different parts of the tail, was considered the TTLOWR. Mice with latencies greater than 2 SDs from the group mean were excluded from further analysis. Ambient temperature was maintained as described above. Rectal temperature was measured after each assay to ensure normothermia. TTLORR at 0.8 MAC, TTLORR at 1.0 MAC, and TTLOWR at 1.3 MAC were determined in different groups of mice of each genotype. Each group was tested with all three anesthetics in a random order. Mice were allowed to recover for a minimum of 24 h before exposure to another anesthetic.

Behavioral Studies of Sensitivity to Intravenous Anesthetics

Ketamine (Sankyo Co., Ltd., Tokyo, Japan), pentobarbital (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), and propofol (AstraZeneca K.K., Osaka, Japan) were injected intraperitoneally after an aspiration test. The anesthetic doses of drugs were selected based on previous reports.[17–19]

Sensitivities to intravenous anesthetics were evaluated using a rating scale as previously described. Each animal was injected with drug and placed in a 2-l glass beaker. At 2-min intervals, the beaker was tilted to an angle of approximately 45° to a horizontal plane, three times, to gently place animals on their backs. The ability of mice to right themselves was noted as anesthetic score according to the rating scale of Boast *et al.*,[20] with minor modifications: a score of 0 indicated normal righting reflex; +1 indicated that the mouse righted itself within 2 s on all three trials (slightly impaired righting reflex); +2 indicated that the latency to righting was greater than 2 s, but less than 10 s at the best response in three trials (moderately or severely impaired righting reflex); and +3 corresponded to the LORR (no righting within 10 s on all three trials). Total anesthetic scores were the sums of all scores recorded after drug injection. The time between LORR (shown as a score of +3) and the time at which mice regained the ability to right themselves (shown as a score of +2) was considered to be the duration of LORR (minutes). The time required to return to a normal righting reflex (shown as a score of 0) was considered to be the recovery time (minutes). Mice with one of these indexes greater than 2 SDs from the group mean were excluded from further analysis. No animal was used for more than two drug treatments, administered at random, and at least 1 week was allowed between treatments for mice to recover. Normothermia was confirmed by measuring rectal temperature immediately after a mouse lost its righting reflex.

Statistical Analysis

The data are presented as mean ± SEM. For statistical analysis of differences in anesthetic sensitivity, we used the Mann–Whitney U test. In all cases, $P < 0.05$ was considered significant.

Results

First, we examined whether the anesthetic actions of volatile anesthetics are altered by α_{1G} T-type calcium channel gene knockout. Mutant mice showed similar sensitivity to the hypnotic and immobilizing effects of all of the volatile anesthetics tested (table 1). No differences were found in the concentrations of isoflurane, halothane, or sevoflurane that were required to cause LORR (LORR ED_{50}) or prevent movement in response to the tail clamp (MAC) in wild-type and mutant mice (table 1).

Table 1. Effects of Volatile Anesthetics on the RR ED_{50} and MAC Values in Mice

Anesthetic	n	RR ED_{50}, % atm	n	MAC, % atm
Isoflurane				
Wild type	14	0.5 ± 0.02	8	1.2 ± 0.05
Mutant	10	0.5 ± 0.03	8	1.3 ± 0.04
Halothane				
Wild type	8	0.7 ± 0.03	12	1.2 ± 0.03
Mutant	8	0.6 ± 0.04	12	1.2 ± 0.02
Sevoflurane				
Wild type	10	1.4 ± 0.05	10	2.8 ± 0.05
Mutant	10	1.5 ± 0.06	10	2.8 ± 0.05

Values are mean ± SEM.

MAC = minimum alveolar concentration; RR ED_{50} = 50% effective concentration for loss of righting reflex.

However, during these assays, we noticed that mutant mice generally required more time to reach an anesthetized state. Therefore, we examined whether the onset of inhalation anesthesia/hypnosis was delayed in mutant mice. For this purpose, mice were exposed to 0.8 and 1.0 MAC concentrations of isoflurane, halothane, or sevoflurane, determined at the beginning of the study, and the TTLORR was measured (figs. 2A–C and table 2).

Exposure to volatile anesthetics produced anesthetic effects/hypnosis in a dose-dependent manner in both groups of mice. As shown in figures 2A–C, LORR occurred at significantly later times in mutant mice exposed to 0.8 MAC of isoflurane, halothane, or sevoflurane or 1.0 MAC of halothane or sevoflurane than in wild-type controls exposed to the same anesthetics. The difference in TTLORR between mutant and wild-type mice was not quite significant ($P = 0.0720$) in the case of 1.0 MAC of isoflurane. Therefore, knockout of the α_{1G} T-type calcium channel results in a delay of onset of inhalational anesthesia in mice.

Genetic inactivation of α_{1G} T-type calcium channels in mice has been shown to result in a moderate slowing of heart rate and atrioventricular conduction.[21] To exclude the possibility of decreased cardiac output as a pharmacokinetic cause of the delayed onset of anesthesia/hypnosis in mutant mice, we measured the time required to cause immobility after exposure to 1.3 MAC concentrations of isoflurane, halothane, and sevoflurane in both groups of mice (fig. 2D and table 2). Because wild-type and mutant mice demonstrated equal anesthetic requirements (MAC) for the loss of tail-clamp reflex, identical times to the onset of immobility in both groups of mice would indicate that equal anesthetic concentrations are reached in the central nervous system (spinal cord)[22] at the same speed. As shown in figure 2D, the TTLOWR in mutant mice after exposure to each anesthetic was not different from that of wild-type controls, thus excluding altered pharmacokinetics as a cause of the prolonged TTLORR in mutant mice.

Because these findings suggested that the normal functioning of α_{1G} channels might be a part of the mecha-
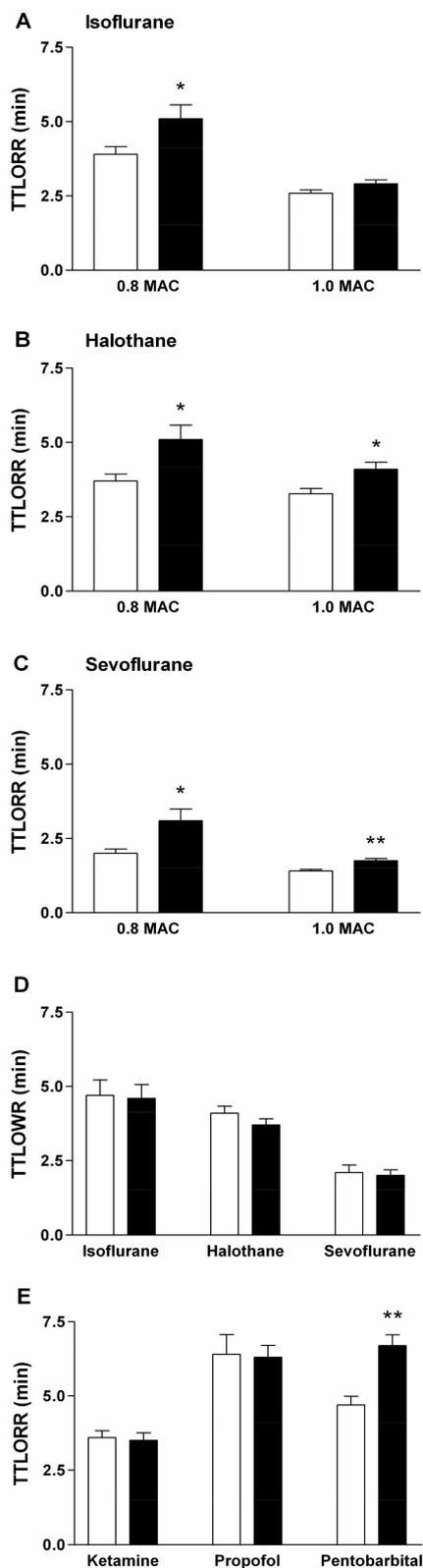
Fig. 2. Effects of volatile (*A–D*) and intravenous (*E*) anesthetics on time to loss of righting reflex (TTLORR) and time to loss of tail withdrawal reflex (TTLOWR) in wild-type (*white bars*) and mutant (*black bars*) mice. Values are mean ± SEM (n = 10–24/group for volatile and n = 11–15/group for intravenous anesthetics). * *P* < 0.05, ** *P* < 0.01, wild-type *versus* mutant mice. MAC = minimum alveolar concentration.

nism by which some general anesthetics initiate anesthesia/hypnosis, we examined whether the onset of LORR was also delayed after administration of intravenous anesthetics. Intraperitoneal injections of ketamine (100 mg/kg) and propofol (180 mg/kg) caused LORR in similar proportions of wild-type and mutant mice (80% and 75%, respectively, after ketamine, and 85% and 86%, respectively, after propofol); no differences in the TTLORR between the two experimental groups of mice after ketamine and propofol were found (fig. 2E). Pentobarbital (40 mg/kg) also produced LORR in similar proportions of wild-type and mutant mice (table 3). However, in contrast to the experiments with ketamine and propofol, mutant mice required significantly more time for LORR to occur, *i.e.*, the TTLORR for pentobarbital was greater in knockout mice (fig. 2E).

Considering this prolongation of TTLORR after pentobarbital, we conducted a more detailed LORR assay to examine whether the other hypnotic properties of pentobarbital were altered in mutant mice as well. Although the duration of LORR (sleeping time) seemed to be longer in mutant mice, the difference did not reach statistical significance (*P* = 0.2936; table 3). It should be also noted that the duration of LORR in mutant mice showed larger variability: minimal and maximal values of the duration of LORR in wild-type and mutant mice were 8–24 and 6–44 min, respectively. Similar to the duration of righting reflex, there were also no differences in total anesthetic scores and recovery times between the two experimental groups (table 3).

Discussion

Here, we examined whether the inhibition of α_{1G} T-type calcium channels reported for anesthetics *in vitro* is relevant to the mechanisms of general anesthesia. Maintained anesthetic state has a hypnotic component and an immobility component defined by the behavioral endpoints of LORR ED_{50} and MAC, respectively. LORR ED_{50} and MAC values for the volatile anesthetics were not altered after α_{1G} calcium channel knockout, indicating that normal functioning of α_{1G} calcium channels is not required for the maintenance of anesthesia.

However, we found that the onset of anesthesia/hypnosis, defined by TTLORR, after exposure to halothane and sevoflurane, is delayed in mutant mice. This suggests that α_{1G} calcium channels are involved in the initiation of anesthesia/hypnosis by some anesthetic agents. In this regard, it should be noted that mice with global or thalamic-specific deletion of α_{1G} calcium channels have been reported to have delayed onset and fragmentation of physiologic sleep.[23] Based on somnographic and electrophysiologic studies, it was concluded that the activation of thalamic α_{1G} calcium channels might be required to block the transmission of arousal signals through the

Table 2. Animals Excluded from the Analysis of TTLORR and TTLOWR after Exposure to Volatile Anesthetics

Animals	Isoflurane, ×MAC			Halothane, ×MAC			Sevoflurane, ×MAC		
	0.8	1.0	1.3	0.8	1.0	1.3	0.8	1.0	1.3
Wild type	1 (16)	1 (24)	1 (12)	1 (16)	0 (24)	0 (18)	0 (12)	0 (16)	0 (10)
Mutant	2 (16)	1 (24)	0 (12)	0 (17)	1 (24)	0 (18)	1 (12)	0 (16)	0 (10)

Values in parentheses indicate the total number of animals in each group.

MAC = minimum alveolar concentration; TTLORR = time to loss of righting reflex; TTLOWR = time to loss of withdrawal reflex.

thalamus to the cerebral cortex to facilitate and stabilize sleep. Accordingly, it is highly possible that volatile anesthetics also use the same endogenous α_{1G} channel-dependent inhibitory mechanism to initiate anesthesia/hypnosis.

In naturally occurring sleep, the activation of α_{1G} calcium channels in thalamic relay nuclei is suggested to transform inhibitory γ-aminobutyric acid–mediated (GABAergic) synaptic inputs into a prolonged inhibition of action potentials.[23] Volatile anesthetics are known to potentiate γ-aminobutyric acid type A (GABA$_A$) receptor function.[24] Therefore, the augmentation of volatile anesthetic-enhanced GABAergic synaptic inputs to thalamic relay nuclei by α_{1G} calcium channels may also serve to interrupt the transmission of arousal signals to the cortex, causing anesthesia/hypnosis. That is, similar to physiologic sleep, the activation of α_{1G} calcium channels might also be required for the induction of inhalational anesthesia.

Although mutant mice showed significantly greater TTLORR after the exposure to 0.8 MAC isoflurane, the difference in TTLORR between wild-type and mutant mice after the exposure to 1.0 MAC of isoflurane was less marked. This finding can be explained by the fact that isoflurane is probably the most potent among volatile agents in terms of inhibition of T-type calcium channels,[10] which occurs at concentrations overlapping its anesthetic range.[11,24] Therefore, it is possible that the augmentation of isoflurane-induced increase in GABAergic tone by α_{1G} calcium channels (and resultant anesthesia/hypnosis) could be disabled or masked because of concomitant inactivation of these channels during the induction of anesthesia by this agent administered at higher concentrations. Such inactivation could occur

Table 3. Effects of Pentobarbital on LORR, Duration of LORR, Total Anesthetic Score, and Recovery Time in Mice

Animals	n	LORR, %	Duration of LORR, min	TAS	Recovery Time, min
Wild type	12 (13/1)	100	13.5 ± 1.3	48.9 ± 2.6	44.8 ± 1.9
Mutant	15 (16/1)	100	20.1 ± 3.2	49.5 ± 3.1	42.1 ± 1.8

Pentobarbital was administered intraperitoneally at 40 mg/kg. Values are mean ± SEM. Values in parentheses indicate the total number of animals/number of excluded animals in each group.

LORR = loss of righting reflex; TAS = total anesthetic score.

before the anesthetic state is reached, thus rendering the contribution of α_{1G} calcium channels to the induction of anesthesia/hypnosis by isoflurane administered at higher concentrations less significant.

All clinically used intravenous anesthetics have been shown to inhibit α_{1G} calcium channels at concentrations exceeding their clinical range. Our results, showing that the onset of LORR is delayed in mutant mice after administration of the barbiturate pentobarbital, suggest that at least some intravenous anesthetics use an α_{1G} calcium channel–dependent mechanism for the initiation of anesthesia/hypnosis. Because the onset of anesthesia/hypnosis in mutant mice was not delayed after administration of ketamine and propofol, these compounds seem to use other mechanisms to cause their hypnotic effect. Ketamine mediates its effects mainly through the blockade of N-methyl-D-aspartate receptors and not through the potentiation of GABA$_A$ receptor function.[24] It selectively depresses neuronal function in parts of the cortex and thalamus, while simultaneously stimulating parts of the limbic system, including the hippocampus—a process termed functional disorganization.[25] Patients anesthetized with ketamine alone seem to be in a cataleptic state, unlike anesthesia with other compounds, which resembles normal sleep.[25] Therefore, the normal onset of ketamine-induced anesthesia/hypnosis in mutant mice, with its distinct properties from other anesthetics, is not surprising. However, the question arises why the onset of LORR is not delayed after administration propofol, which is a primarily GABAergic agent like pentobarbital.

One possible explanation comes from the fact that the hypnotic action of pentobarbital and propofol might be mediated *via* different central nervous system nuclei involved in the generation of sleep. Indeed, pentobarbital and propofol differ in the extent of their inhibitory actions on the tuberomammillary nucleus, which represents another target site critically involved in causing sleep.[26] It was demonstrated that systemic or local injections of the GABA$_A$ receptor antagonist gabazine into the tuberomammillary nucleus were considerably less potent in attenuating the LORR induced by pentobarbital than the LORR induced by propofol.[26] Therefore, it is reasonable to suppose that to cause anesthesia/hypnosis, propofol might act preferentially on the tuberomammil-

lary nucleus, whereas pentobarbital might be more active on other nuclei, such as those that project to thalamocortical neurons and initiate the α_{1G} calcium channel–dependent inhibition of arousal signals traveling to the cortex.

Another possible explanation is best considered in light of a recently advocated theory of narcosis.[27] According to this theory, anesthetics are hypothesized to realize their effects through numerous interaction points within the thalamocortical–corticothalamic–reticulothalamic loops leading to thalamocortical hyperpolarization, block of information processing, and unconsciousness/hypnosis. In fact, as well as potentiating GABAergic signaling, volatile anesthetics and pentobarbital can produce a direct hyperpolarization effect on thalamocortical neurons.[28-30] Propofol also hyperpolarizes thalamic relay neurons, but probably does this indirectly by increasing the excitability of reticular thalamic neurons, which provide the major source of feedforward GABAergic inhibition of thalamocortical cells.[31] This excitatory effect of propofol is mediated through the blockage of the small-conductance calcium-activated potassium (SK) channels expressed in reticular thalamic neurons.[31] Intriguingly, recent evidence also suggests that any induction of a hypnotic state by propofol that involves hyperpolarization within thalamocortical circuits will primarily engage the cortical neuron component.[32] This propofol-induced hyperpolarization of cortical pyramidal neurons also seems to be a GABA-independent effect, realized through the selective inhibition of hyperpolarization-activated cyclic nucleotide–modulated (HCN) channels containing HCN1 subunits.[32]

Animal studies have demonstrated that onset of physiologic sleep happens because thalamocortical neurons become hyperpolarized.[33] As mentioned above, this process seems to be defective in mutant mice with thalamic or global deletion of α_{1G} T-type calcium channels.[23] However, the onset of sleep is only mildly delayed when these channels are deleted from cortical pyramidal neurons,[23] which normally exhibit abundant expression of this subunit.[3] In parallel to physiologic sleep, genetic inactivation of α_{1G} T-type calcium channels in the thalamus might impinge on the direct hyperpolarizing effects of volatile anesthetics and pentobarbital in thalamocortical cells and thereby postpone the onset of anesthesia/hypnosis. On the other hand, analogous to the less affected physiologic sleep pattern in mutant mice with cortical deletion of the α_{1G} T-type calcium channels, the direct hyperpolarizing effect of propofol on pyramidal neurons and the resultant propofol-induced sleep-like hypnotic state might also be insignificantly affected by the genetic inactivation of α_{1G} T-type calcium channels. These considerations, combined with probable distinctive effects of propofol on thalamocortical sensory information processing mediated through

non-GABAergic ionic mechanisms, may provide another plausible explanation for our results.

Although gene knockout has become a powerful tool with which to understand the neural basis of anesthesia, a number of confounds exist in such studies, and these should be carefully considered before drawing any definitive conclusions.[34] In regard to this study, it is possible that the lack of α_{1G} T-type calcium channels was compensated for by up-regulation of other calcium channel subunits. However, several considerations allow us to think that such compensation probably does not occur after α_{1G} calcium channel knockout. Site-specific genetic ablations reduce the likelihood of confounding changes and the study by Anderson *et al.*[23] indicated that both global and thalamus-specific deletion of α_{1G} calcium channel have similar disruptive effects on physiologic sleep. Also, in mouse heart, the expression of other calcium channels does not compensate for the lack of α_{1G} calcium channels.[21] These findings suggest that the results of our study may be attributed to the lack of targeted gene and not to compensatory effect caused by genetic manipulation.

In knockout studies it is also important to differentiate between the pharmacokinetic and pharmacodynamic effects of a gene inactivation. T-type calcium channels are also expressed in pacemaker and conduction cells of the mammalian heart.[4] Electrocardiograms and intracardiac recordings document bradycardia due to the slowing of the sinoatrial node pacemaker activity and atrioventricular conduction in mice lacking α_{1G} T-type calcium channels, although the reduction in mean heart rate in freely moving mutant mice is moderate (10%).[21] However, considering the property of volatile anesthetics to prolong atrioventricular conduction time in a concentration-dependent manner,[35] there is a possibility that compromised cardiac output could be responsible for the delayed induction of anesthesia/hypnosis in mutant mice. If altered pharmacokinetics were the cause of delayed onset of anesthesia/hypnosis in mutant mice, one would expect the differences in induction times (TTLORR) to be more pronounced at higher inhaled concentrations of volatile anesthetics. However, this was not the case with a single anesthetic; rather, the prolongation of TTLORR in mutant mice at 0.8 MAC was greater than that at 1.0 MAC. Furthermore, the difference in induction times would be more pronounced at the even higher 1.3 MAC concentrations used to evaluate TTLOWR (time to immobility). Given the fact that anesthetic requirements for MAC were unchanged in mutant mice, identical TTLOWR in both experimental groups indicate that equal concentrations of volatile anesthetics were reached at the anesthetic target sites in the central nervous system (spinal cord)[22] at the same time points, despite some slowing of heart rate after genetic inactivation of α_{1G} T-type calcium channels. Finally, it should be noted that autonomic regulation of

heart rate is preserved in mutant mice: They display no cardiac arrhythmias and have normal systolic, diastolic, and mean arterial pressures.[21] These findings together with our observations allow us to exclude altered pharmacokinetics as a possible cause of delayed onset of anesthesia/hypnosis after α_{1G} calcium channel knockout. Although we have not formally checked the brain concentrations of pentobarbital, its altered pharmacokinetics are unlikely to be the cause of delayed onset of LORR in mutant mice. First, except for the prolonged TTLORR, mutant mice demonstrated a time course of pentobarbital anesthesia that was otherwise similar to that in wild-type animals; the recovery times were not different between two experimental groups. Second, a supposedly greater susceptibility of mutant mice to the depressant effect of pentobarbital on atrioventricular conduction is also not likely as a possible cause of delayed onset of anesthesia/hypnosis, because such depressant effects would be equal or even more pronounced with propofol, which is reported to have more marked negative dromotropic effect that barbiturates[36]; however, this was not the case.

There is another secondary consequence of α_{1G} calcium channel knockout that could have caused the difference between the two genotypes. Mutant mice may have increased homeostatic sleep drive due to fragmentation and shortening of sleep.[23] Sleep deprivation has been shown to reduce TTLORR in animals given propofol or isoflurane, and to prolong the time to recovery from anesthesia.[37] Thus, there is a possibility that the same phenomenon could have accounted for the delay in TTLORR observed in our experiments. If this is the case, sleep deprivation would probably have resulted in increased susceptibility to all tested anesthetics, but TTLORR was not changed in our study after the administration of propofol and ketamine to mutant mice. Also, as documented for pentobarbital, mutant mice recovered after anesthesia at times similar to wild-type controls. This argues against an increased propensity to sleep as a possible nonspecific cause for our results. Of note, mutant mice have reduced total time of non–rapid eye movement sleep,[23] but not the duration of pentobarbital-induced LORR. This probably illustrates one of the fundamental differences between anesthesia and sleep: Although the former may share a common mechanism with non–rapid eye movement sleep, the presence of the anesthetic prevents one from being awake.[27] At the same time, greater variability in the duration of LORR after pentobarbital administration in mutant mice probably parallels the phenomenon of sleep fragmentation with frequent arousal bouts documented after α_{1G} calcium channel knockout.[23]

Recent evidence suggests that anesthetics act on brain structures known to participate in sleep generation.[26,27,38] This raises the possibility that anesthesia exerts its effects by interacting with the neuronal networks normally involved in sleep.[39,40] Therefore, sleep and anesthesia might be more similar than was previously thought. Our results with mutant mice support this idea and suggest that the induction of anesthesia/hypnosis with inhalational and intravenous anesthetics requires activation of the α_{1G} calcium channel-dependent brain mechanisms involved in the naturally occurring shift from a state of vigilance to sleep.

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