1 mg/kg succinylcholine was used for blockade of neuromuscular transmission and facilitation of tracheal intubation. Sugammadex (16 mg/kg) was administered 3 min after rocuronium administration. Mean times to recovery of T1 to 10 and 90% were significantly faster in the rocuronium–sugammadex group as compared with the succinylcholine group. Hence, they concluded that reversal of profound high-dose rocuronium-induced neuromuscular block (1.2 mg/kg) with 16 mg/kg sugammadex was significantly faster than spontaneous recovery from 1 mg/kg succinylcholine. This report implies that the rocuronium–sugammadex combination may be useful for inducing muscle paralysis during ECT. Our study indicated possible equipotent effects of rocuronium- and succinylcholine-induced neuromuscular block for muscle relaxation.

Certain other factors should be considered while analyzing our results.

First, we previously showed that the onset and duration of muscle relaxants were greatly influenced by cardiac output before injection [13]. Hence, the onset and duration of muscle relaxation in each of our patients may have been affected by their individual cardiac outputs.

Second, in this study, sugammadex was infused after the end of the seizure. Puhringer et al. [12] reported that the timing of administration of sugammadex might influence the reversal of profound rocuronium-induced neuromuscular blockade. Thus, it is possible that our results were affected by the timing of administration of sugammadex.

The mechanism of the longer seizure duration with ECT following rocuronium–sugammadex administration as compared to that with SCC administration is unknown. Small differences in the hyperventilation status before electrical stimulation between the two groups might have some effect on seizure duration [1]. Another possibility is that the number of sessions of ECT might have affected the seizure duration because of improvement in the depressive condition resulting from ECT [1].

The dose of 0.6 mg/kg of rocuronium used in this study was half the dosage used by Lee et al. [2]. However, a dose of 16 mg/kg of sugammadex was used as the neuromuscular antagonist in this study. Reportedly, a dose of at least 4–8 mg/kg of sugammadex is needed for recovery from deep neuromuscular blockade, indicated as a post-tetanic count of 1–2 on the TOF monitor [14]. We believe that more profound neuromuscular blockade was induced in our study group by administration of sugammadex as compared to this previous study. In addition, we were afraid of the risk of recurarization with use of a small dose of sugammadex. Hence, a dose of 16 mg/kg of sugammadex was used as the neuromuscular antagonist in this study.

We measured variables only twice during ECT. With subsequent ECT sessions, patients require larger doses of propofol to achieve unconsciousness due to improvement in the depressive condition induced by ECT. Hence, the propofol dosage could have greatly influenced seizure duration and hemodynamic changes induced by ECT in this study. In addition, improvement in the depressive condition by ECT could have led to the anti-depressant agent being changed, which could also have affected the hemodynamic changes induced by ECT.

For unknown reasons, there were some differences in the time to recovery of T1 to 10 and 90% and the time to the first spontaneous breath between case one and the others. One possible speculation for this difference is that case 1 exhibited differential effects to the non-depolarizing neuromuscular agents because of undetectable degeneration, demyelination or axon loss in the motor nerve ending of the neuromuscular junction and infarction or atrophy of the skeletal muscle. This could partly explain why almost twice the time was needed for the recovery of T1 to 10 and 90% in this patient. Another possibility is that the onset and duration of muscle relaxants are greatly influenced by cardiac output before injection [13] be responsible for these differences observed in case 1.

Although the cost of sugammadex may preclude its routine use for ECT, rocuronium–sugammadex may be useful for muscle relaxation during ECT in patients in whom the use of succinylcholine is contraindicated, such as those with severe osteoporosis, amyotrophic lateral sclerosis and a history of neuroleptic malignant syndrome [7].

In conclusion, we demonstrated the potential efficacy of rocuronium–sugammadex as an alternative to succinylcholine for muscle relaxation during ECT.

Conflict of interest None.

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The influence of manipulations to alter ambient GABA concentrations on the hypnotic and immobilizing actions produced by sevoflurane, propofol, and midazolam

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ABSTRACT

Recent studies have suggested that extrasynaptic $GABA_A$ receptors, which contribute tonic conductance, are important targets for general anesthetics. We tested the hypothesis that manipulations designed to alter ambient GABA concentrations (tonic conductance) would affect hypnotic (as indicated by loss of righting reflex, LORR) and immobilizing (as indicated by loss of tail-pinch withdrawal reflex, LTWR) actions of sevoflurane, propofol, and midazolam. Two manipulations studied were 1) the genetic absence of glutamate decarboxylase (GAD) 65 gene (GAD65−/−), which purportedly reduced ambient GABA concentrations, and 2) the pharmacological manipulation of GABA uptake using GABA transporter inhibitor (NO-711). The influence of these manipulations on cellular and behavioral responses to the anesthetics was studied using behavioral and electrophysiological assays. HPLC revealed that GABA levels in GAD65−/− mice were reduced in the brain (76.7% of WT) and spinal cord (68.5% of WT). GAD65−/− mice showed a significant reduction in the duration of LORR and LTWR produced by propofol and midazolam, but not sevoflurane. NO-711 (3 mg/kg, ip) enhanced the duration of LORR and LTWR by propofol and midazolam, but not sevoflurane. Patch-clamp recordings revealed that sevoflurane (0.23 mM) slightly enhanced the amplitude of tonic GABA current in the frontal cortical neurons; however, these effects were not strong enough to alter discharge properties of cortical neurons. These results demonstrate that ambient GABA concentration is an important determinant of the hypnotic and immobilizing actions of propofol and midazolam in mice, whereas manipulations of ambient GABA concentrations minimally alter cellular and behavioral responses to sevoflurane.

1. Introduction

The GABAergic system in the central nervous system (CNS) is a key target of general anesthetics (Mihic et al., 1997; Sonner et al., 2003; Rudolph and Antkowiak, 2004; Hemmings et al., 2005; Franks, 2006). Two types of GABAergic inhibition are known; a phasic form (phasic inhibition) regulating neural excitability via the activation of postsynaptic $GABA_A$ receptors by intermittent GABA release from presynaptic terminals, and a persistent tonic form (tonic inhibition) generated by continuous activation of extrasynaptic $GABA_A$ receptors by low concentrations of ambient GABA (Brickley et al., 1996). Growing evidence suggests that tonic inhibition mediated by extrasynaptic $GABA_A$ receptors might contribute to the actions of intravenous anesthetics such as propofol (Bai et al., 2001; Bieda and MacIver, 2004). These extrasynaptic $GABA_A$ receptors have different pharmacological and kinetic properties compared with synaptic $GABA_A$ receptors, as a result of the distinct subunit compositions (Glykys and Mody, 2007). Given that extrasynaptic $GABA_A$ receptors respond to low ambient levels of GABA, manipulations of ambient GABA concentrations may affect cellular and behavioral responses to general anesthetics.

Two manipulations studied were 1) the genetic absence of glutamate decarboxylase (GAD) 65 gene (GAD65−/−), and 2) the pharmacological manipulation of GABA uptake using GABA transporter inhibitor. GAD is the only synthetic enzyme responsible for the conversion of L-glutamic acid to GABA. The brain contains two forms of GAD, which differ in molecular size, amino acid sequence,

Abbreviations: ACSF, artificial cerebrospinal fluid; GABA, γ-aminobutyric acid; mIPSC, miniature inhibitory postsynaptic current; GAD, glutamate decarboxylase; GAT, GABA transporter; LORR, loss of righting reflex; LTWR, loss of tail-pinch withdrawal response; NO-711, 1-[2-[[(Diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride ($C_{21}H_{22}N_2O_3 \cdot HCl$).

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antigenicity, cellular and subcellular locations, and interaction with the GAD cofactor pyridoxal phosphate (Erlander et al., 1991). The 67-KDa isoform (GAD67) is found mainly in the cell body, whereas GAD65 is localized to the nerve terminal and is reversibly bound to the membrane of synaptic vesicles (Namchuk et al., 1997). GAD65−/− mice remain viable without apparent anatomical deficits and postsynaptic $GABA_A$ receptor density is unchanged (Kash et al., 1997), although the survival rate of GAD65−/− mice was slightly reduced with age, largely due to spontaneous seizures (Stork et al., 2000). As a result of reduced GABAergic tone, GAD65−/− mice appear to show increased anxiety levels (Kash et al., 1999; Kubo et al., 2009a), different sensitivity to pentobarbital (Kash et al., 1999), and hyperalgesia to thermal, but not chemical, stimulation (Kubo et al., 2009b). On the other hand, inhibition of GABA uptake and/or metabolism is a strategy for enhancing ambient GABA concentrations. GABA is cleared from the synaptic cleft by specific, high-affinity, sodium- and chloride-dependent transporters, which are thought to be located on presynaptic terminals and surrounding glial cells, i.e., four distinct GABA transporters, GAT-1, GAT-2, GAT-3 and BGT-1 (Borden, 1996). NO-711, a potent and selective GAT-1 inhibitor, was used because GAT-1 is responsible for the majority of neuronal GABA transport.

We have reported that sevoflurane enhances GABAergic inhibition (Nishikawa and MacIver, 2001; Nishikawa and Harrison, 2003; Nishikawa et al., 2005), suggesting that $GABA_A$ receptor is one of the plausible molecular targets. In addition, several targets have been also proposed for inhalational general anesthetics; glycine receptors (Mascia et al., 1996), two-pore-domain potassium channels (Sirois et al., 2000), NMDA receptors (Sonner et al., 2003), HCN channels (Chen et al., 2005), and some subtypes of sodium channels (Wu et al., 2004), whereas a specific point mutation in $GABA_A$ receptor is critical for propofol and etomidate (Jurd et al., 2003). These data suggest that the relative contributions of GABAergic inhibition to *in vivo* anesthetic actions are different between sevoflurane and intravenous anesthetics. We first tested the hypothesis that genetic and pharmacological manipulations to alter ambient GABA concentrations would affect loss of righting reflex (LORR), a surrogate measure of hypnosis, and loss of tail-pinch withdrawal reflex (LTWR), a measure of immobilization, produced by sevoflurane, propofol, and midazolam. We then studied the influence of these manipulations on *in vitro* sevoflurane actions on membrane properties of frontal cortical layer V neurons using patch-clamp methods. The present study provides evidence that genetic and pharmacological manipulations to alter ambient GABA concentrations (tonic conductance) affect the response to propofol and midazolam, but minimally affect the actions of sevoflurane.

2. Materials and methods

2.1. Mice

All animal procedures and protocols used in this study were approved by the Animal Care Committee of Gunma University Graduate School of Medicine (protocol # 05-71) and performed through NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The generation of glutamate decarboxylase 65 (GAD65) knockout mice used in the present study was described by Yanagawa et al. (1999) and Yamamoto et al. (2004). In brief, we designed a targeting vector to disrupt most of the open reading frame by inserting an in-frame stop codon in the exon 3. The linearized targeting vector was introduced by electroporation into E14.1 embryonic stem (ES) cell derived from strain 129/Ola mice, and we obtained ES cell clones carrying the GAD65 targeted mutation through homologous recombination. The correctly targeted ES cells were injected into C67BL/6J mouse (CLEA Japan, Inc., Tokyo, Japan) blastocysts to make chimeras. The chimeric male mice were mated with female C57BL/6J, and germ-line transmission was achieved. The resultant GAD65 heterozygous (+/−) mice were backcrossed for more than ten generations onto the C57BL/6J background. Wild-type (+/+) and knockout (−/−) littermates were produced from heterozygous mating pairs. GAD65−/− mice were viable and fertile and gross behaviors appeared to be normal without apparent anatomical deficits. Adult (12–16-weeks old) male WT mice and GAD65−/− mice weighing 23–27 g were used for experiments. Mice were group-housed in a pathogen-free transgenic facility, and water and food were available *ad libitum*. None of the animals were used for more than two experiments and at least 1 week was allowed for the mice to recover.

2.2. Measurement of neurotransmitter contents

For analysis of neurotransmitter tissue content, WT mice and GAD65−/− mice at 12 weeks of age were sacrificed by decapitation under deep sevoflurane anesthesia. Tissue samples of the whole brain and the whole spinal cord were removed quickly and tissue weight was measured. The tissue was added to 3–5 ml of saline (saline volume was approximately ten folds of tissue weight), and then homogenized in phosphate-buffered saline (PBS) containing 0.2% protease inhibitor using a polytron homogenizer (24,000 rpm, 15 s, 2–3 times). Following removal of cell debris by centrifugation at 3000 rpm (20 min, 4 °C), the supernatant (500 μl), which was added to sulfosalicylic acid (750 μl), was centrifuged again at 3000 rpm (20 min, 4 °C). The supernatant after pH adjustment was analyzed using high-performance liquid chromatography (HPLC) and fluorescence detection. HPLC was performed by the company (SRL, Tokyo, Japan). Neurotransmitter content (nmol/g) was calculated as follows: measured neurotransmitter concentration (nmol/ml) × saline volume added (ml)/tissue weight (g).

2.3. Behavioral assays for intravenous drugs

Loss of righting reflex (LORR) was used as a surrogate measure for hypnosis. Each animal was received an intraperitoneal (ip) injection of propofol (Maruishi Pharmaceuticals Co, Ltd., Osaka, Japan) or midazolam (Astellas Pharma Inc., Tokyo, Japan) with a volume of 10 μl/g of body weight, and then placed on their backs in a chamber (20 × 28 × 15 cm). The ability to right themselves was evaluated as described (Kubo et al., 2009a). Because we have previously reported that GAD65−/− mice showed altered responses to propofol (100 mg/kg, ip) (Kubo et al., 2009a), propofol (125 mg/kg, ip) was tested in the present study. Midazolam (50 mg/kg, ip) was used as described previously (Quinlan et al., 1998). Mice were judged to have lost this reflex when unable to right itself within 10 s. The time from ip injection of the drug to LORR was considered as the latency, and the time between the LORR and the time mice regained the ability to right themselves within 2 s was considered the duration of LORR. Loss of tail-pinch withdrawal response (LTWR) was used as a surrogate measure for immobilization (Quasha et al., 1980). A surgical spring clip (6 mm in size, Applied Medical, CA, USA) was placed at the base of an animal's tail for 5 s.

Vehicle solutions for behavioral studies were as follows: propofol, lipofundin MCT/LCT 10% (B. Braun Melsungen AG, Melsungen, Germany); midazolam, saline. An intraperitoneal injection of lipofundin MCT/LCT 10% (10 μl/g) alone had no hypnotic/analgesic effect on mice behavior ($n = 5$). NO-711 hydrochloride (Sigma-Aldrich Japan, Tokyo, Japan), a potent and selective GAT-1 inhibitor that cross the blood–brain barrier (Borden et al., 1994), was diluted in sterile saline and injected 20-min prior to experiments (a volume of 10 μl/g of body weight). Other drugs were purchased from Sigma–Aldrich Chemicals (Tokyo, Japan).

2.4. Behavioral studies of sensitivity to sevoflurane

Mice were placed into a sealed Plexiglas chamber (32 × 32 × 22 cm), warmed by heating pads from below. After 20-min equilibration period with a chosen concentration of sevoflurane (0.5–5.0% atm, Maruishi, Osaka, Japan) delivered via an anesthetic-specific vaporizer (Sevotec 5, Ohmeda, UK) with fresh air flow at a rate of 3.0 l/min, a blinded observer scored the mice for LORR and LTWR in a quantal fashion. Sevoflurane concentration was continuously controlled by the infrared gas analyzer (BP-508, Nippon Colin Co. Ltd., Tokyo, Japan). In LORR assays, mice were judged to have lost righting reflex when unable to right itself within 10 s. In LTWR assays, movement to tail-pinch was tested by the placement of the surgical clip at the base of an animal's tail for 5 s. If any movement to tail-pinch was detected, the concentration of sevoflurane was increased for another 20-min equilibration period, and the response was tested again. The concentration at which the mouse lost its tail-pinch reflex was noted. Sevoflurane concentration also was confirmed by gas chromatograph analysis (GC-4000, GL Sciences Inc., Tokyo, Japan) of samples drawn from the chamber.

2.5. Electrophysiology

The methods of brain slice electrophysiology were described previously (Nishikawa et al., 2005; Ishizeki et al., 2008). Briefly, mice were decapitated under deep isoflurane anesthesia, and the brain was then removed and immediately immersed in a cold (1–4 °C) modified Ringer solution, comprised of 234 mM sucrose; 2.5 mM KCl; 1.25 mM NaH_2PO_4; 10 mM $MgSO_4$; 0.5 mM $CaCl_2$; 26 mM $NaHCO_3$; and 11 mM glucose saturated with 95% O_2 and 5% CO_2. A block of tissue

containing the frontal cortex was quickly dissected out and glued to a DTK-1000 vibratome tray (Dosaka EM, Tokyo, Japan) using oxygenated cold modified Ringer solution. Slices (500 μm) were cut from the brain and then kept in the pre-chamber (Brain Slice Chamber System; Harvard Apparatus, Holliston, MA) filled with artificial cerebrospinal fluid (ACSF) consisting of (in mM), 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 11 glucose, bubbled with 95% O_2 and 5% CO_2 at room temperature (22–24 °C). Slices were allowed 1 h for recovery in the pre-chamber, which was designed to keep 8–12 slices viable for several hours.

Slices were transferred to a recording chamber (1.0 ml in volume, Brain Slice Chamber System, Harvard Apparatus, Inc., Holliston, MA) perfused with an oxygenated ACSF at a rate of approximately 3 ml/min. Patch electrodes were made from borosilicate thin-walled capillaries (GDC-1.5, Narishige Co., Ltd., Tokyo, Japan). Recording electrodes (5–7 MΩ) were filled with Cs_2SO_4-based solution (Cs_2SO_4 110 mM, TEA 5 mM, $CaCl_2$ 0.5 mM, $MgCl_2$ 2 mM, EGTA 5 mM, HEPES 5 mM and MgATP 5 mM; pH 7.2) to investigate spontaneous IPSCs at a holding potential of 0 mV. The liquid junction potential in these conditions was approximately 9 mV, and all data presented were corrected using this value. K-gluconate solutions (K-gluconate 100 mM, EGTA 10 mM, HEPES 40 mM, $MgCl_2$ 5 mM, ATP 2 mM, GTP 0.3 mM, pH 7.25.) were used for current clamp recordings, so the impermeable ion (gluconate) would not contribute to anesthetic-induced changes in resting membrane potential or current–voltage relations. Whole cell patch-clamp recordings were made from visualized layer V pyramidal neurons in the frontal cortex using an upright Axioskop2 FS plus microscope (Zeiss, Jena, Germany). The magnified image was collected by an intensified CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) with contrast enhancement. The image of neurons was displayed on a video monitor, and glass patch pipettes were visually advanced using a micromanipulator (MWO3, Narishige Co., Ltd., Tokyo, Japan) through the slice to the surface of the neuron. Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA) was used for whole cell recordings. Whole cell currents were filtered at 2–5 kHz and digitized at 10 kHz (Digidata 1322A, Axon Instruments Inc.) and stored on a Pentium-based PC and paper recorder for later analysis. The GABAergic nature of the synaptic currents was verified by applying the $GABA_A$ receptor antagonist, picrotoxin (PIC). Series resistances were between 10 and 25 MΩ, and were then compensated approximately 80%. Recordings were performed at room temperature (22–24 °C).

In some experiments using WT mice, NO-711 (3 mg/kg) was injected intraperitoneally 20 min before decapitation. It was confirmed, 10 min later, that NO-711 prolonged the latency to jumping or licking responses in the hot-plate test (53 °C) (Kubo et al., 2009b), and then brain slices were made as described above. The effects of NO-711 in the slice remained effective for several hours, since the amplitude of tonic inhibition was larger than that of WT mice without NO-711 injection ($n = 5$). Thus, *in vitro* experiments with NO-711 were performed within 4 h after making slice preparations. In separate experiments, NO-711 was directly added to the ACSF solution that was bathing the slice in the recording chamber. However, in this case, it was difficult to determine the concentration that should be used to match the dose injected in behavioral studies. Judging from the influence on tonic inhibition, we estimated that 1–2 μM NO-711 were relevant concentrations.

2.6. Data analysis

Data acquisition and analysis were performed with pCLAMP software version 8.1 (Axon instruments Inc., Union City, CA) and IGOR Pro version 5.0 (WaveMetrics, Lake Oswego, OR). Synaptic currents were defined as current deflections with a fast rising phase and a relatively slower decay phase. The rise time was defined as time interval between 10% and 90% of the peak amplitude and synaptic currents having the rise time <2 ms were included for analysis. The amplitude of synaptic current was measured from the initial inflection point (not from the baseline) to the peak, to avoid the effects of summation on amplitude distribution. Threshold-level crossing were set at approximately three folds of baseline noise, which was measured during the period of no detectable events. As a result, synaptic currents larger than 6 pA in the amplitude were counted for analysis. This definition eliminated the infrequently observed single channel events or synaptic currents with slow rise time, but successfully detected most IPSCs. The decay phase was fitted with a single exponential curve and a time from peak to 1/e was defined as the decay time.

2.7. Application of sevoflurane to slices and concentration measurement

Artificial cerebrospinal fluid solution at room temperature was bubbled with a carrier gas (95% O_2, 5% CO_2) passing through a calibrated commercial vaporizer (Sevotec 5; Ohmeda, BOC Health Care, West Yorkshire, United Kingdom) at the designated concentration, and was applied to the recording chamber using a gravity-feed and vacuum system. High-quality polytetrafluorethylene was used for tubing and valves to minimize loss of volatile anesthetic and drug binding. Sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) concentrations used for this study were 2.8% (clinically relevant concentration for mice) and 5.0% (high dose for mice). To determine the actual aqueous concentrations of sevoflurane in the submerged recording chamber for each concentration used, aliquots of the solution were taken from the recording chamber and filled into airtight glass containers for gas chromatographic measurements as described previously (Ishizeki et al., 2008). In brief, aliquots of the solution were directly taken from the recording chamber for gas chromatographic measurements. The peak of sevoflurane was observed approximately 3 min after injection, and the area under the curve was measured. The aqueous sevoflurane concentration was calculated by comparing to that of sevoflurane standard solution (1.0 mM), in which 20 μl of sevoflurane was dissolved in ethanol (100 ml). The final aqueous concentration of 2.8% sevoflurane was 0.23 ± 0.01 mM ($n = 5$), and 5.0% was 0.41 ± 0.01 mM ($n = 5$).

Although sevoflurane is administered to humans in the gas phase at body temperature (37 °C), *in vitro* electrophysiological experiments using sevoflurane were carried out at room temperature. In general, gas-phase potencies are reported to be temperature-dependent, increasing markedly with decreasing temperatures (Franks and Lieb, 1998). Procedures using gas-phase EC_{50} concentrations for room temperature experiments can thus result in overdosing with the *in vitro* preparations. Franks and Lieb (1996) have reported that the upper estimate of mammalian minimum alveolar concentration values for sevoflurane expressed as free aqueous concentration in saline is 0.33 mM. Taking these data into considerations, sevoflurane 0.23 mM was used as a clinically relevant concentration and 0.41 mM as a relatively high concentration.

2.8. Statistics

Results are expressed as mean ± SD. The results were analyzed by using Student's *t*-test or one way analyses of variance (ANOVA). Post hoc comparisons between the individual groups were performed by means of the Tukey test. Statistical significance between curves fitted to LORR and LTWR data was performed by comparing EC_{50} values via *t*-test. The level of statistical significance was set at $P < 0.05$ in all tests.

3. Results

3.1. GABA levels in the brain and the spinal cord are reduced in GAD65−/− mice

We measured the GABA content in the whole brain and spinal cord in GAD65−/− mice at 12–16-weeks old. GAD65−/− mice showed a significant reduction in GABA levels in the brain (76.7% of WT, $P < 0.001$, $n = 6$ each, Fig. 1A) and the spinal cord (68.5% of WT, $P < 0.001$, $n = 5$ for WT and $n = 6$ for GAD65−/− mice, Fig. 1B). Although a compensatory mechanism involving the balance between inhibitory and glutamatergic excitatory neurotransmission might have been expected, the difference in glutamate and glycine levels did not reach statistical significance when calculating mean values from experiments on five or six animals, respectively.

3.2. Ambient GABA levels are altered in GAD65−/− mice and in WT mice following blockade of GAT-1 transporter

Because the major anesthetic targets are believed to be cortical neurons and thalamic neurons (Franks, 2006), $GABA_A$ receptor-mediated miniature IPSCs (mIPSCs) were recorded from the frontal cortex layer V pyramidal neurons at 0 mV, close to the reversal potential of EPSCs, using Cs_2SO_4-based internal solutions (Fig. 2A). Tetrodotoxin (TTX, 1 μM) was used to block sodium channels that give rise to action potentials. Under these conditions, glutamate-mediated EPSCs were negligible. These observations were further confirmed by applying a GABAergic antagonist, picrotoxin (PIC, 50 μM or 100 μM, Pitler and Alger, 1992). Because both doses produced a similar baseline shift, PIC (50 μM) was used in following experiments. These neurons were identified using IR-DIC microscopy by their large (>20 μm diameter) pyramidal shaped cell bodies with long apical dendrites extending toward the pial surface. The mean amplitude of mIPSCs was unchanged in GAD65−/− mice or in WT with NO-711 injection (12.9 ± 3.5 pA in WT mice, 11.9 ± 4.7 pA in GAD65−/− mice, and 13.9 ± 3.5 pA in WT mice with NO-711, $n = 10$ each). The rise time of mIPSCs was also similar among groups (WT mice, 0.8 ± 0.2 ms, $n = 10$; GAD65−/− mice, 0.9 ± 0.3 ms, $n = 10$, WT mice with NO-711 (ip), 0.8 ± 0.3 ms, $n = 10$).

Ambient GABA levels were then evaluated by measuring the amplitude of tonic conductance of layer V pyramidal neurons in the

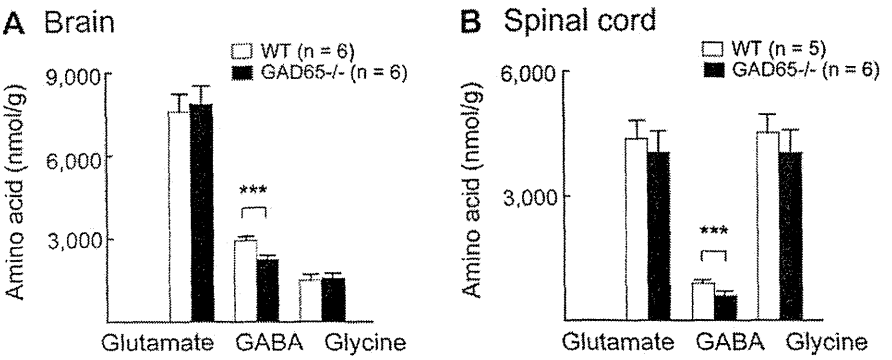

Fig. 1. GABA contents of GAD65−/− mice are reduced in the brain (A) and the spinal cord (B). Neurotransmitter contents were analyzed by high-performance liquid chromatography (HPLC). (A) In the whole brain, GABA contents were significantly reduced in GABA65−/− mice ($n = 6$, $P < 0.001$, t-test) compared with those of WT mice ($n = 6$). Data bars show mean ± SD. (B) In the spinal cord, GABA contents were also significantly reduced in GAD65−/− mice ($n = 6$, $P < 0.001$, t-test). Note that glutamate and glycine contents were unaffected by GAD65 gene knockout in the brain and the spinal cord. Data bars show mean ± SD.

frontal cortex of mice. The effects of PIC on the holding current were examined in the absence of the anesthetic. Although PIC (50 μM, 5 min) produced an inward shift in all groups, the amplitude of tonic current was slightly but significantly reduced in GAD65−/− mice (13.3 ± 5.5 pA in GAD65−/− slices, $n = 12$; 24.4 ± 6.5 pA in WT slices, $n = 11$, $P < 0.001$, Fig. 2B). On the other hand, the tonic conductance was significantly increased in WT mice treated with a GAT-1 inhibitor NO-711 (3 mg/kg, ip) (34.5 ± 7.8 pA, $n = 12$, $P < 0.01$ vs. WT slices, Fig. 2B). These data provide indirect evidence that ambient GABA levels in the frontal cortex are altered by genetic and pharmacological manipulations used in the present study.

3.3. Behavioral responses to sevoflurane are unchanged in GAD65−/− mice

We next examined whether the anesthetic sensitivity to sevoflurane is altered by GAD65 gene knockout. The LORR assay was performed to examine the anesthetic sensitivity of mice to sevoflurane. Mice were judged to have lost this reflex when unable to right itself within 10 s. The dose–response curves for the ED_{50} determination for LORR are presented in Fig. 3A. The calculated ED_{50} values for LORR were 1.24% in WT mice ($n = 14$) and 1.26% in GAD65−/− mice ($n = 14$). Thus, GAD65 gene knockout did not affect anesthetic sensitivity to sevoflurane. LTWR was used as a surrogate measure for immobilization (Quasha et al., 1980). Sevoflurane concentration was increased and the ED_{50} value for LTWR was determined. The calculated ED_{50} values for LTWR were 2.07% in WT mice ($n = 21$) and 2.04% in GAD65−/− mice ($n = 21$). These data suggest that hypnotic and immobilizing actions of sevoflurane are unaffected by GAD65 gene knockout and resulting reduction in GABA content (Fig. 1).

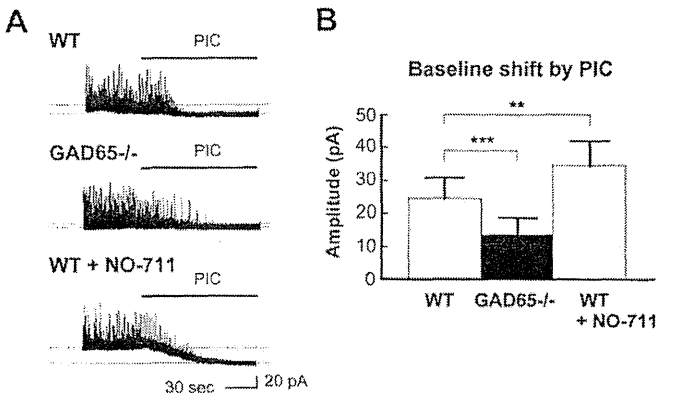

Fig. 2. The effects of genetic and pharmacological manipulations on the amplitude of tonic inhibition in cortical layer V neurons. Neurons were voltage-clamped at 0 mV using Cs_2SO_4-based internal solutions. (A) Representative traces of mIPSCs from WT slices (top), GAD65−/− slices (middle), and WT with NO-711 slices (bottom). These mIPSCs were completely blocked after application of picrotoxin (PIC, 50 μM) in all groups. (B) Baseline shift produced by PIC (50 μM) was significantly smaller in cortical neurons of GAD65−/− slices (***$P < 0.001$ vs. WT), and larger in cortical neurons of WT with NO-711 slices (**$P < 0.01$ vs. WT). Data bars show mean ± SD.

3.4. Behavioral response to propofol and midazolam are diminished in GAD65−/− mice

We then compared anesthetic sensitivity to propofol and midazolam, a positive allosteric modulator of $GABA_A$ receptors. The latency to LORR produced by propofol (125 mg/kg, ip) was significantly prolonged in GAD65−/− mice (188.9 ± 23.6 s, $n = 10$, $P < 0.01$,) than that of WT mice (150.0 ± 27.5 s, $n = 10$). The duration of LORR produced by propofol (125 mg/kg, ip) was significantly reduced in GAD65−/− mice (1568.0 ± 689.0 s, $n = 10$, $P < 0.05$) than that of WT mice (2263.0 ± 533.6 s, $n = 10$, Fig. 3B *left*). Similarly, the duration of LTWR produced by propofol (125 mg/kg, ip) was significantly reduced in GAD65−/− mice ($n = 8$, $P < 0.01$, Fig. 3B *right*). As shown in Fig. 3C, the duration of LORR produced by midazolam (50 mg/kg, ip) was significantly reduced in GAD65−/− mice (2498.4 ± 1351.0 s, $n = 9$, $P < 0.05$) than that of WT mice (3564.0 ± 854.0 s, $n = 21$) without affecting the latency to LORR ($P = 0.60$ between genotypes). In addition, the duration of LTWR produced by midazolam (50 mg/kg, ip) was significantly reduced in GAD65−/− mice ($n = 8$ each, $P < 0.05$).

3.5. Behavioral responses to sevoflurane are unchanged by GABA transporter 1 inhibition

We then tested whether the selective GAT-1 inhibitor, NO-711, would affect LORR produced by sevoflurane, propofol and midazolam. Because enhanced GABA concentration by NO-711 may produce hypnotic actions in the absence of the anesthetics, we first confirmed that NO-711 at low doses (1 or 3 mg/kg, ip) had no effect on righting reflex in both genotypes. Righting reflex was slightly impaired at 6 mg/kg (ip) and severely impaired at high doses (>10 mg/kg). These data suggest that NO-711 alone can induce hypnosis in a dose-dependent manner by enhancing tonic inhibitions.

NO-711 (3 mg/kg, ip) did not affect dose–response relationship of LORR produced by sevoflurane (Fig. 4A). The calculated ED_{50}

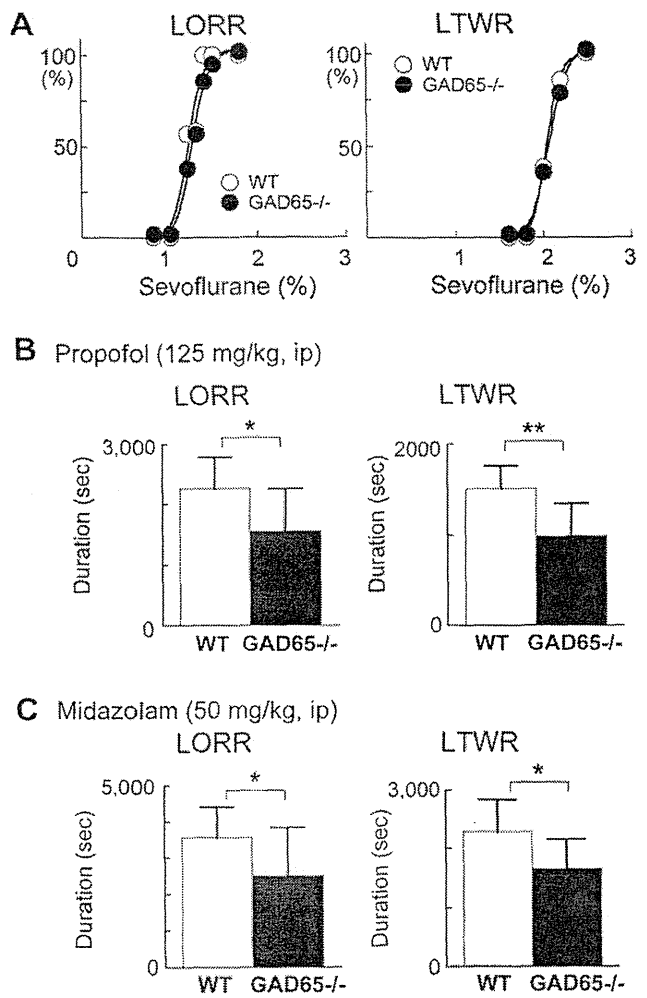

Fig. 3. The effects of reduced GABA contents by GAD65 gene knockout on behavioral responses to sevoflurane, propofol, and midazolam. (A) Dose–response curves for the determination of the ED_{50} value for loss of righting reflex (left) and for loss of tail-pinch withdrawal response (right) produced by sevoflurane. Data presented are the fraction of mice that did not respond, i.e., failed to right themselves on the LORR or failed to move in response to the tail clamp vs. sevoflurane concentration. The fitted curves were generated using a weighted sum of least-squares method (Kaleida-Graph version 3.5, Reading, PA) to a Hill equation: LORR (%) = $[drug]^{nH}/([drug]^{nH} + [ED_{50}]^{nH})$; where LORR (%) is a percentage of LORR, [drug] is the anesthetic concentration, and nH is the Hill coefficient. EC_{50} did not differ between genotypes for either assay. (B) The duration of LORR and LTWR were compared between WT mice and GAD65−/− mice. A significant reduction in the duration of LORR (*$P < 0.05$ between genotypes, $n = 10$) and LTWR (**$P < 0.01$) was observed in GAD65−/− mice. (C) A significant reduction in the duration of LORR and LTWR was observed in GAD65−/− mice (*$P < 0.05$ between genotypes, $n = 9$). Data bars show mean ± SD.

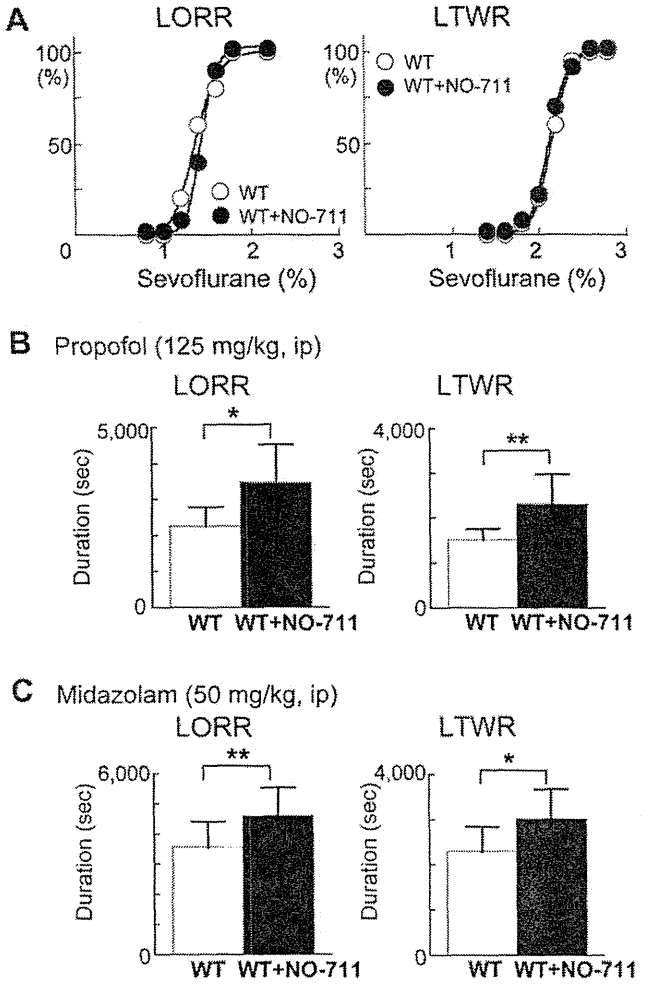

Fig. 4. The effects of increased GABA contents by NO-711 on behavioral responses to sevoflurane, propofol and midazolam. (A) Pre-injection of NO-711 (3 mg/kg, ip) had little effect on the dose–response relationship of LORR (left) and LTWR (right) produced by sevoflurane. (B) NO-711 (3 mg/kg, ip) significantly increased the duration of LORR ($n = 8$, *$P < 0.05$) and LTWR (**$P < 0.01$) produced by propofol (125 mg/kg, ip). (C) The effects of NO-711 (3 mg/kg, ip) on the duration of LORR and LTWR produced by midazolam (50 mg/kg, ip). NO-711 increased the duration of LORR ($n = 7$, **$P < 0.01$) and LTWR (*$P < 0.05$). Data bars show mean ± SD.

values for LORR were 1.28% in WT mice ($n = 10$) and 1.36% in GAD65−/− mice ($n = 10$). In LTWR assays, NO-711 (3.0 mg/kg, ip) had no effect on immobilizing actions produced by sevoflurane. The calculated ED_{50} values for LTWR were 2.15% in WT mice ($n = 13$) and 2.12% in WT mice with NO-711 (3 mg/kg, ip) ($n = 13$).

3.6. *Behavioral responses to propofol and midazolam are enhanced by GAT-1 inhibition*

NO-711 (3 mg/kg, ip) significantly increased the duration of LORR produced by propofol (125 mg/kg, ip) from 2263.0 ± 533.6 s ($n = 10$) to 3471.7 ± 1076.1 s ($n = 8$, $P < 0.05$, Fig. 4B) in WT mice without affecting the latency to LORR ($n = 8$, $P = 0.66$). NO-711 (3 mg/kg, ip) also increased the duration of LTWR ($P < 0.01$) produced by propofol (125 mg/kg, ip, Fig. 4B *right*). NO-711 (3.0 mg/kg) increased the duration of LORR produced by midazolam (50 mg/kg, ip) from 3564.0 ± 854.0 s to 4601.0 ± 951.0 s ($n = 7$, $P < 0.01$, Fig. 3C *left*) without affecting the latency to LORR ($n = 7$, $P = 0.89$). NO-711 (3 mg/kg, ip) increased the duration of LTWR ($n = 7$, $P < 0.05$) produced by midazolam (50 mg/kg, ip, Fig. 4C *right*).

3.7. *Sevoflurane-induced enhancement of phasic and tonic inhibition*

Intravenous anesthetic such as propofol and thiopental strongly enhance both phasic and tonic inhibition (Bai et al., 2001; Bieda and MacIver, 2004; Bieda et al., 2009). However, little is known about the effects of sevoflurane on phasic and tonic components of GABAergic inhibition. Although basal inhibitory synaptic transmission was normal in GAD65−/− mice (Kubo et al., 2009a; Tian et al., 1999) and WT mice with NO-711, sevoflurane enhanced GABAergic inhibition in cortical pyramidal neurons. Sevoflurane (0.23 mM) significantly increased the frequency of mIPSCs in WT

mice (180.1% of WT control, $n = 8$, $P < 0.001$), GAD65−/− mice (168.9% of GAD65−/− mice control, $n = 8$, $P < 0.001$), and WT with NO-711 (ip) (195.0% of NO-711 control, $n = 8$, $P < 0.001$). There was no group difference in the frequency in the presence of sevoflurane (0.23 mM, ANOVA). Sevoflurane (0.23 mM) also increased the decay time of mIPSCs in WT mice (241.1% of WT control, $n = 8$, $P < 0.001$), GAD65−/− mice (232.9% of GAD65−/− mice control, $n = 8$, $P < 0.001$), and WT with NO-711 (ip) (267.0% of NO-711 control, $n = 8$, $P < 0.001$). There was no group difference in the decay time in the absence or presence of sevoflurane (ANOVA). In addition, sevoflurane (0.23 M) produced a relatively small but significant baseline shift in I_{hold} in all groups (Fig. 5A). The amplitudes of baseline shift produced by sevoflurane were 13.1 ± 5.0 pA in WT mice ($n = 8$, $P < 0.001$ vs. control), 11.2 ± 3.9 pA in GAD65−/− mice ($n = 8$, $P < 0.001$ vs. control), and 14.9 ± 5.9 pA in WT mice with NO-711 (ip) ($n = 8$, $P < 0.001$ vs. control, Fig. 5B). These sevoflurane effects were reversible after washout of the anesthetic.

A specific $GABA_A$ receptor antagonist, SR95531 (1 μM), selectively blocks only synaptic $GABA_A$ receptors that generate IPSCs (Yamada et al., 2007), while PIC blocks both synaptic and extrasynaptic tonic conductances (Bieda and MacIver, 2004). SR95531 does not affect GABA-transaminase or GAD activities. To examine the relative contributions of synaptic components in GABAergic inhibition, we tested the effects of SR95531 (1 μM) on mIPSCs in the presence of sevoflurane (0.23 mM). SR95531 (1 μM, 5 min) abolished mIPSCs in all groups but had little effect on baseline holding current (WT slices, $n = 5$; GAD65−/− slices, $n = 4$; WT slices with NO-711, $n = 5$). Prolonged application (20 min) produced a small baseline shift in I_{hold} in all group, these changes did not reach statistical significance because of their variability.

3.8. Sevoflurane-induced depression of pyramidal neuron excitability

Whole cell recordings were obtained from visually identified layer V pyramidal neurons located within the medial PFC. Resting membrane potentials were −71.3 ± 2.6 mV ($n = 15$) in WT slices, −70.6 ± 3.5 mV ($n = 14$) in GAD65−/− slices, and 71.7 ± 3.9 mV ($n = 15$) in WT slices with NO-771. The majorities of neurons were typically silent at rest and were held for periods for >15 min. Although bath application of sevoflurane (0.23 mM, 20 min) produced a small hyperpolarization in all groups, but these effects did not reach statistical significance because of cell to cell variability. Sevoflurane (0.41 mM, 20 min) significantly hyperpolarized the neuron (−5.3 ± 2.5 mV in WT slices, $n = 8$; −6.3 ± 3.6 mV in GAD65−/− slices, $n = 8$; −7.3 ± 4.0 mV in WT slices with NO-711, $n = 8$).

In response to supra-threshold currents, some pyramidal neurons exhibited modest spike frequency accommodation, other neurons responded to current injection with relatively regular inter-spike intervals. Sevoflurane-induced neural depression was compared by measuring the number of APs to depolarizing current injection, as a measure of effects on intrinsic excitability. We focused on pyramidal neurons with relatively regular inter-spike intervals (Fig. 6A). In WT mice, sevoflurane (0.23 mM, 20 min) did not change action potential discharge responses to depolarizing

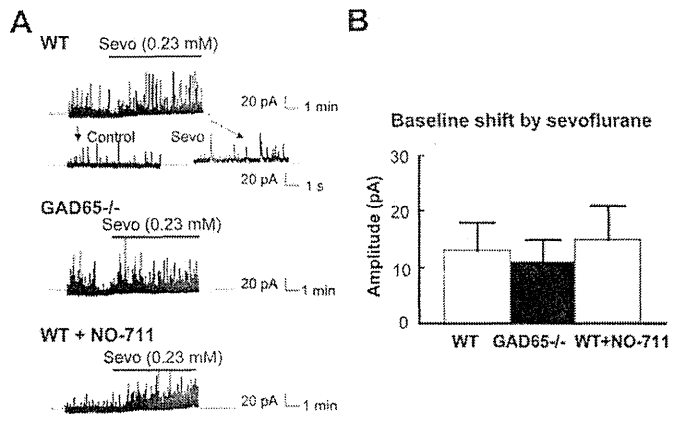

Fig. 5. The effects of sevoflurane (0.23 mM) on mIPSCs recorded from layer V pyramidal neurons n WT mice, GAD65−/− mice and WT mice with NO-711 injection. (A) Sample traces show spontaneous IPSCs recorded in normal ACSF solution in the presence of TTX (1 μM). Neurons were voltage-clamped at 0 mV using Cs_2SO_4-based internal solutions, and mIPSCs were recorded from WT mice (top), GAD65−/− mice (middle), and WT mice with NO-711 injection (bottom). Note that sevoflurane (0.23 mM, 20 min) increased the frequency and prolonged the decay phase, and also shifted the baseline. The effects of sevoflurane on the baseline currents reached plateau 5–10 min after sevoflurane application. (B) Sevoflurane (0.23 mM) slightly but significantly produced a baseline shift in all groups ($P < 0.05$ vs. control, $n = 8$). There was no significant difference among groups. Data bars show mean ± SD.

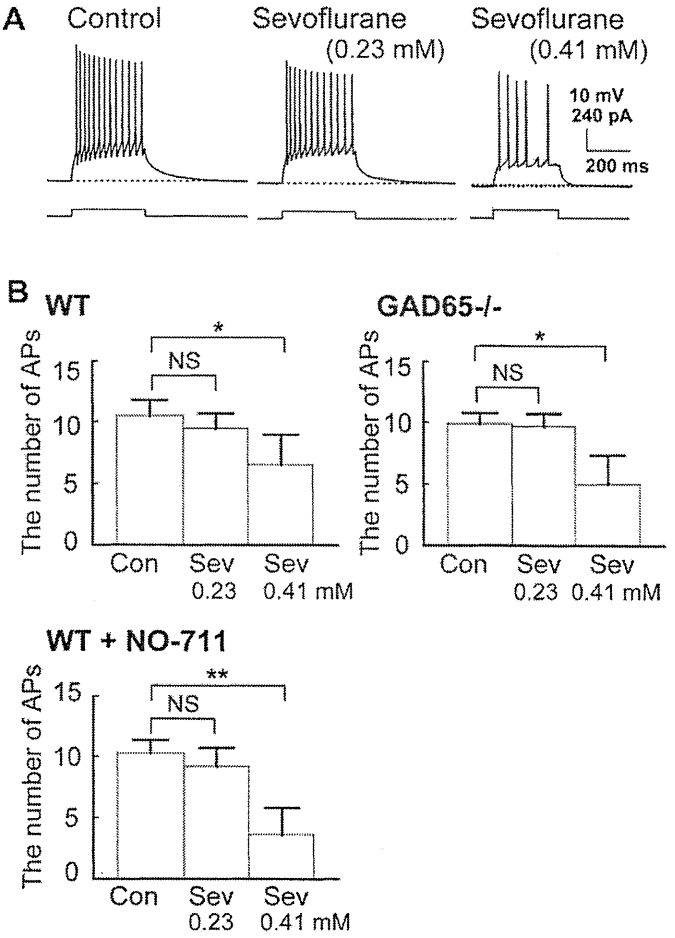

Fig. 6. The effects of sevoflurane on voltage responses to depolarizing current injection to pyramidal neurons with non-accommodating response. Recordings were made in whole cell current clamp using K-gluconate as the internal solution. (A) Representative examples of sevoflurane (0.23 mM) effects on AP discharge activity of cortical neurons in WT mice. The number of AP discharge induced by current injection (120 pA, 300 ms) was not altered by sevoflurane at clinically relevant concentration (0.23 mM), but inhibited by high concentration of sevoflurane (0.41 mM). (B) The effects of sevoflurane on AP discharge were compared among WT mice, GAD65−/− mice, and WT with NO-711 mice. NO-711 (3 mg/kg) were injected intraperitoneally 20 min prior to brain slice preparation. In WT mice, sevoflurane at 0.41 mM, but not 0.23 mM, inhibited the number of APs ($P < 0.05$ vs. control, $n = 8$, ANOVA). Similarly, sevoflurane at 0.23 mM had no effect on AP discharge activity ($n = 8$ for GAD65−/− mice and $n = 9$ for WT + NO-711 mice, ANOVA). Data are presented as mean ± SD.

current in pyramidal neurons. The number of APs was significantly depressed when sevoflurane (0.41 mM) was applied to the chamber (Fig. 6B). In WT mice, sevoflurane at 0.41 mM significantly depressed the number of APs from 10.5 ± 1.3 to 6.5 ± 2.4 ($n = 10$, $P < 0.05$ vs. control, ANOVA-Tukey). Similar results were observed in GAD65−/− mice and WT mice with NO-711 (ip). Although sevoflurane at 0.23 mM had no effect on neuronal excitability of pyramidal neurons in GAD65−/− mice ($n = 9$) and WT mice with NO-711 (ip) ($n = 9$), sevoflurane at 0.41 mM significantly depressed the number of APs in GAD65−/− mice ($n = 9$, $P < 0.05$ vs. control, ANOVA-Tukey) and in WT mice with NO-711 (ip) ($n = 9$, $P < 0.01$ vs. control, ANOVA–Tukey). The membrane input resistance in pyramidal neurons was very similar in all groups under control conditions. Superfusion of sevoflurane 0.41 mM, but not 0.23 mM, reversibly reduced input resistance to a similar degree in all groups (91.3 ± 3.4% of control in WT slices, $n = 5$; 93.3 ± 4.3% of control in GAD65−/− slices, $n = 5$; 89.8 ± 5.4% of control in WT slices with NO-711, $n = 5$).

In separate experiments, NO-711 was directly added to the ACSF solution that was bathing the slice in the recording chamber. The effects of NO-711 on sevoflurane-induced depression of pyramidal neuron excitability were basically similar to those observed with intraperitoneally injected NO-711 (3 mg/kg). In the presence of NO-711 (2 μM), sevoflurane (0.23 mM) had little effect on neuronal excitability of pyramidal neurons ($n = 6$), whereas sevoflurane (0.41 mM) significantly depressed the number of APs ($n = 5$, $P < 0.01$ vs. control, ANOVA–Tukey).

4. Discussion

GAD65−/− mice appeared to show normal sensitivity to sevoflurane despite a 20–30% reduction in GABAergic inhibitory tone, whereas they showed reduced sensitivity to propofol and midazolam. In contrast, enhanced GABA concentrations by NO-711 prolonged the duration of LORR and LTWR by propofol and midazolam, but not sevoflurane. Sevoflurane enhanced tonic inhibition of layer V cortical neurons; however, these effects were not strong enough to alter discharge properties of cortical neurons. These data show that genetic and pharmacological manipulations designed to alter ambient GABA concentrations differentially regulate hypnotic and immobilizing actions of general anesthetics.

4.1. Reduced ambient GABA concentrations in GAD−/− mice

GAD65−/− mice appeared to show partial reductions in GABA concentrations in the whole brain and whole spinal cord. However, HPLC data include synaptic vesicular, intracellular, and extracellular (ambient) GABA concentrations. Patch-clamp studies confirmed that the amplitude of tonic inhibition was significantly reduced in GAD65−/− mice and that this reduction was relevant to HPLC data. Given that the amplitude of tonic inhibition reflects the ambient GABA concentration (Semyanov et al., 2004), we conclude that ambient GABA concentrations are reduced in GAD65−/− mice, and that reduced ambient GABA concentrations are responsible for altered behavioral responses to propofol (Kubo et al., 2009a) and midazolam.

Although a compensatory mechanism involving the balance between inhibitory and glutamatergic excitatory neurotransmission might have been expected, glutamate and glycine levels were unchanged. As a result, the ratio of excitatory to inhibitory neurotransmitter levels actually increased in GAD65−/− mice. It has been reported that the survival rate of GAD65−/− mice was thus significantly reduced, largely due to spontaneous seizures; only 2–3% of WT mice died before postnatal 25 weeks, whereas 25% of GAD65−/− mice died during the same period (Stork et al., 2000). To minimize the possibility of a generalized increase in CNS excitability and behavioral abnormality during experiments, 12–16-week old mice were used in our study. In fact, we have previously confirmed that excitatory synaptic transmission in hippocampal slices was normal in GAD65−/− mice, and that the survival rate was almost similar between genotypes at this stage (Kubo et al., 2009a).

4.2. Reduced sensitivity to propofol and midazolam, but not sevoflurane, in GAD65−/− mice

Our data are consistent with previous reports showing that GAD65−/− mice had reduced sensitivity to pentobarbital, which is known to potentiate GABAergic inhibition (Kash et al., 1999). Although the molecular and cellular factors underlying differences in anesthetic sensitivity are currently unknown, one possibility is that sevoflurane may have multiple molecular targets contributing to *in vivo* hypnotic actions (Mascia et al., 1996; Sirois et al., 2000; Sonner et al., 2003; Chen et al., 2005; Wu et al., 2004). If so, the present data lead us to believe that relatively small changes in ambient GABA concentrations are insufficient to alter behavioral responses to sevoflurane. To support this notion, sevoflurane has been shown to depress excitatory synapses in synaptosomes (Moe et al., 2003), the brainstem (Stucke et al., 2005), and the hippocampus (Ishizeki et al., 2008), whereas propofol has little effect on excitatory synapses in cortical neurons (Kitamura et al., 2003), the hippocampus (Kubo et al., 2009a), and the spinal cord (Takazawa et al., 2009). Together, available data suggest that imbalance in excitatory and inhibitory neurotransmitter contents in the brain significantly affect hypnotic and immobilizing actions of propofol and midazolam.

It must be emphasized, however, that the influence of GAD65 knockout is relatively small in terms of anesthetic sensitivity. In fact, hypnotic actions could be elicited in GAD65−/− mice with the use of higher concentrations of propofol and midazolam. The most likely interpretation of these findings is that other molecular targets may mediate behavioral responses to these agents. In this context, mutations in postsynaptic $GABA_A$ receptors have appeared to modulate *in vivo* propofol actions (Jurd et al., 2003). The mutation of asparagine to methionine (N265M) in the β3 subunit greatly reduces the ability of propofol and etomidate to cause LORR and eliminates their abilities to prevent response to painful stimuli, but has little effect on the actions of volatile anesthetics. In contract, the $GABA_A$ α1 subunit (S270) is a critical determinant that influences a variety of behaviors in the mouse in view of volatile anesthetic sensitivity (Homanics et al., 2005). These data show that postsynaptic $GABA_A$ receptors play more important roles with *in vivo* anesthetic potencies.

4.3. Increased extracellular GABA concentrations by GABA transporter 1 inhibition

The actions of synaptically released GABA are terminated by uptake into presynaptic terminals and surrounding glial cells via Na^+-dependent transporters. Molecular biological studies have cloned four subtypes of mouse GABA transporter (GAT): GAT-1, GT-2, GAT-3, and GAT-4 (Borden, 1996). NO-711 is a potent and selective GABA uptake inhibitor that exhibits the highest affinity for human GAT-1. Hybridization signals for GAT-1 mRNA have been observed over many regions of the rat brain, including the retina, olfactory bulb, neocortex, ventral pallidum, hippocampus, and cerebellum (Durkin et al., 1995), indicating that NO-711 induces an increase in endogenous GABA concentrations at many brain regions. However, the degree of increased GABA concentrations produced by NO-711 is difficult to determine. We estimated changes in GABA concentrations using two methods. First, we

confirmed physiologically that the amplitude of tonic conductance in cortical pyramidal neurons of brain slices from WT mice with NO-711 (3 mg/kg, ip) was greater than that of WT mice without apparent changes in IPSC amplitude, indicating that the tonic current is not a summation of IPSCs, but is instead attributable to continuous $GABA_A$ receptor activation by endogenous GABA. Second, we confirmed behaviorally that NO-711 (1.0–10 mg/kg, ip) dose-dependently increased the latency in the hot-plate test in WT mice and GAD65−/− mice (Kubo et al., 2009b). Judging from these data, NO-711 (1.0 mg/kg, ip) to GAD65−/− mice reinstated GABA levels similar to WT mice, because the latency was similar between groups. High-dose NO-711 (6.0–10 mg/kg) produced slight hypnotic actions in the absence of anesthetics (Kubo et al., 2009b). Together, we believe that NO-711 (3.0 mg/kg, ip) enhances ambient GABA contents without producing hypnotic properties.

One critical issue is the selectivity of the manipulations that were used to alter tonic but not phasic inhibition. Although the manipulations may well alter ambient GABA concentrations and thereby alter tonic inhibition, they may also alter phasic inhibition. Concerning this issue, we have previously confirmed that GAD65 gene knockout reduced tonic inhibition without affecting phasic inhibition (IPSCs) (Kubo et al., 2009a). Tian et al. (1999) have also reported that the frequency of sIPSCs was intact in retinal ganglion cells in GAD65−/− mice. Although we do not know the source of compensation for the synthesis of GABA being released, deleting GAD65 gene may have little effect on the GABA content of spontaneously released synaptic vesicles.

4.4. Molecular targets of general anesthetics: synaptic and extrasynaptic $GABA_A$ receptors

Growing evidence suggests that tonic $GABA_A$ receptors may be more important mediators of anesthetic-induced neural depression than phasic $GABA_A$ receptors (Orser, 2006). This concept is largely based on findings that tonic $GABA_A$ receptors are very sensitive to low concentrations of anesthetics. The continuous, non-desensitizing nature of tonic $GABA_A$ receptors could be expected to contribute to a larger charge transfer or shunting inhibition. The present study suggests that this notion may be true for some, but not all, types of general anesthetics. In contrast to the strong depressant effects produced by propofol (Bieda and MacIver, 2004) and thiopental (Bieda et al., 2009), actions of sevoflurane (0.23 mM) were not strong enough to alter discharge properties of layer V cortical neurons. In support of our data, isoflurane appears to enhance phasic $GABA_A$ inhibition, but does not enhance tonic $GABA_A$ receptors to depress synaptically evoked discharge of hippocampal CA1 neurons (Bieda et al., 2009). In addition, halothane at clinical concentrations (0.35 mM) has only minimal depressant effects on the postsynaptic membrane excitability of CA1 pyramidal cells (Sonner et al., 2003). AP discharge in response to depolarizing current injection is not altered by halothane, nor is the threshold, rise time or amplitude of spikes altered. Furthermore, Ogawa et al. (2011) have examined the effects of sevoflurane (0.3 mM) on extrasynaptic GABA receptors in mechanically dissociated hippocampal CA1 neurons. GABA (1 μM)-induced currents were enhanced by sevoflurane (138% of control), suggesting that extrasynaptic GABA receptors may contribute to the enhancement of the inhibitory responses to some degree. All over the result suggest that the role of ambient GABA concentrations (tonic conductance) may be different in volatile vs. intravenous anesthetics.

In this context, we are interested in the influence of ambient GABA contents on amnestic actions of anesthetics, because it has been shown that $GABA_A$ receptor α5 subunit null mutant mice had very little etomidate-induced amnesia, long-term potentiation, and tonic current augmentation (Cheng et al., 2006).

5. Conclusions

The present study provides *in vivo* evidence that genetic and pharmacological manipulations to alter ambient GABA concentrations have significant effects on the hypnotic and immobilizing actions of propofol and midazolam, whereas these manipulations minimally alter cellular and behavioral responses to sevoflurane.

Acknowledgments

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ORIGINAL ARTICLE

Comparison of recovery times from rocuronium-induced muscle relaxation after reversal with three different doses of sugammadex and succinylcholine during electroconvulsive therapy

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Abstract
Purpose This study was conducted to compare recovery times from rocuronium-induced muscle relaxation after reversal with three different doses of sugammadex with succinylcholine during electroconvulsive therapy (ECT).
Methods Seventeen patients who were scheduled to undergo ECT were studied. Anesthesia was induced by use of propofol (1.0 mg/kg) followed by either succinylcholine (SCC) (1 mg/kg) or rocuronium (0.6 mg/kg). Assisted mask ventilation was initiated with 100% oxygen. After T1 was assessed as being zero by neuromuscular monitoring, an electroshock stimulus was applied bilaterally. Patients receiving rocuronium were infused with 16, 8, or 4 mg/kg sugammadex immediately after the seizure stopped to reverse the muscle relaxation. Neuromuscular monitoring was continued until recovery of the train-of-four ratio to 0.9 at the tibial nerve in the leg. The times to recovery of T1 to 10 and 90% with both relaxants were compared.
Results The time to recovery of T1 to 90% after 16 mg/kg sugammadex was shorter than that in subjects treated with SCC ($p = 0.046$), whereas that after 4 mg/kg sugammadex was longer than that in subjects treated with SCC (SCC group: 429 ± 65 s, 16 mg/kg sugammadex group: 387 ± 63 s*, 8 mg/kg sugammadex group: 462 ± 66 s, 4 mg/kg sugammadex group: 563 ± 45 s*,#; *$p < 0.05$ compared with SCC, #$p < 0.01$ compared with 16 mg/kg sugammadex).
Conclusions This study demonstrates the efficacy of rocuronium–sugammadex as an alternative to SCC for muscle relaxation during ECT, and indicates that 8 mg/kg sugammadex produces equally rapid recovery from rocuronium muscular relaxation compared with spontaneous recovery from 1 mg/kg SCC during ECT.

Keywords Electroconvulsive therapy · Muscle relaxant · Rocuronium · Sugammadex · Succinylcholine

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Introduction

Succinylcholine (SCC) is commonly used as a muscle relaxant during electroconvulsive therapy (ECT) because of its rapid onset and short duration of action [1]. However, SCC has many side effects, for example myalgia, a small increase in plasma potassium, and increase in intra-gastric and intra-ocular pressures [1].

Sugammadex has recently been introduced as a fast-acting, selective relaxant-binding agent that was designed to rapidly reverse rocuronium-induced neuromuscular block. Lee et al. [2, 3] reported that reversal of profound rocuronium (1.0–1.2 mg/kg)-induced neuromuscular block with a large dose of sugammadex (16 mg/kg) was significantly faster than spontaneous recovery from SCC. Previously, we showed the potential benefit of using rocuronium (0.6 mg/kg)–sugammadex (16 mg/kg) as an alternative to SCC (1 mg/kg) for muscle relaxation during ECT [4]. A large dose of rocuronium (1.0–1.2 mg/kg) is usually not needed for muscle relaxation during ECT, as shown in our previous study [4] and that of others [1]. Hence, we speculated that a slightly smaller dose of sugammadex would be required for equally rapid recovery from 0.6 mg/kg rocuronium-induced muscle relaxation as from relaxation with 1 mg/kg SCC.

The purpose of this study was to determine the dose of sugammadex that would produce an equal recovery time

from 0.6 mg/kg rocuronium-induced muscle relaxation as from spontaneous recovery from relaxation with 1 mg/kg SCC during ECT.

Materials and methods

Informed consent was obtained from patients or their families. All protocols were approved by the local Institutional Clinical Study Committee and the Institutional Review Board. Seventeen patients who were scheduled to undergo ECT were studied. None of the patients had a history of cardiovascular, hepatic, renal, or neuromuscular disease, or were obese (BMI >35).

Anesthetic management

All patients underwent at least 10 sessions of ECT (three times per week at 1 or 2-day intervals). To avoid induction of the parasympathetic reflex, the patients received atropine (0.01 mg/kg IM) 30 min before the ECT procedure.

Data measured during the procedure included blood pressure (BP), heart rate, oxygen saturation (SpO_2; measured by pulse oximetry on the left or right index finger), end-expiratory partial pressure of carbon dioxide (end-tidal CO_2:$PetCO_2$) at the nostrils (Capnomac Ultima; Datex, Helsinki, Finland) and electrocardiogram (ECG; lead II). Measurements were initiated before ECT and were terminated at the end of the procedure.

Anesthesia was induced by use of propofol (1.0 mg/kg intravenously over 5 s), followed by either SCC (1 mg/kg intravenously) or rocuronium (0.6 mg/kg intravenously) over 5 s, followed by a 10-ml saline bolus. Assisted mask ventilation was initiated with 100% oxygen. After T1 was assessed as being zero by neuromuscular monitoring, an electroshock stimulus was applied bilaterally for 5 s.

$PetCO_2$ was maintained at 30–35 mmHg and the SpO_2 value was maintained above 98% by manual mask assistance throughout the therapy. Patients who received rocuronium were infused with 16, 8, or 4 mg/kg sugammadex with a 10-ml saline bolus immediately after the seizure stopped.

During the first and second ECT sessions, we confirmed that 1 mg/kg propofol and 1 mg/kg SCC could provide adequate anesthetic conditions and muscle relaxation to all patients. In addition, the intensity of the ECT stimulus required to achieve a minimum seizure duration of more than 20 s was determined during these sessions.

All patients received 1 mg/kg SCC as the muscle relaxant agent for first three of the subsequent ECT sessions and 0.6 mg/kg rocuronium during the next three sessions. In the remaining sessions (from 7 to 10 or 12 sessions) 1 mg/kg SCC was used as the muscle relaxant agent. When rocuronium was used as the muscle relaxant, patients received one of three sugammadex dosages (16, 8, or 4 mg/kg), with a 10-ml saline bolus immediately after the seizure stopped, the dose of sugammadex to be used in a particular session being decided by a lottery system. Administration of sugammadex was in a non-blinded manner. Electroencephalographic (EEG) seizure duration was recorded by a two-channel EEG after administration of the electrical stimulus.

Neuromuscular assessment

Neuromuscular monitoring was performed using the TOF-watch SX (Organon, Roseland, NJ, USA). The tibial nerve in the leg was supramaximally stimulated at the inferolateral aspect of the medial malleolus with square pulses of 0.2-ms duration, delivered as train-of-four (TOF) pulses, at intervals of 15 s. The resulting contractions of the great toe muscles were quantified by an acceleromyographic monitor. Calibration was performed and baseline responses were recorded after propofol administration and before muscle relaxant administration. A 50-Hz titanic stimulus was applied for 5 s and followed after 1 min by TOF stimulation every 15 s. When the response to TOF stimulation was stable, calibration and supramaximal stimulation were ensured by the in-built calibration function. Neuromuscular monitoring was continued until recovery of the TOF ratio to 0.9. Following the protocol of our previous study [4], we compared the time to recovery of T1 to 10 and 90% between relaxants. T1 was zero in all patients when sugammadex was administered. We used recovery of T1 to 10% (instead of TOF ratio) for its simplicity, its common usefulness between depolarizing and non-depolarizing relaxants, and because the TOF ratio has uncertain significance with a single dose of SCC.

Patients were also assessed for clinical signs such as the time interval between the first spontaneous breath and administration of muscle relaxant and the time to opening of eyes to verbal commands.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Before the study was started, sample size was evaluated. The sample size was calculated on the basis of the hypothesis that recovery of T1 to 10% with 4 mg/kg sugammadex would be prolonged to 60 s compared to that with SCC [4]. The sample size provided 80% power to detect a 20% difference between 4 mg/kg sugammadex and the SCC groups with a 5% probability of a type II error. A paired *t* test was used for comparison of the two groups. For multiple comparisons, one-way factorial ANOVA and the Bonferroni test were used for the

comparison. Values of $p < 0.05$ were considered statistically significant.

Calculations were performed by Stat View 5.0 software (Abacus Concepts, Berkeley, CA, USA).

Results

Patient age, height, and weight were 58 ± 14 years, 157 ± 7 cm, 57 ± 10 kg, respectively. Seven of the 17 patients were male.

Table 1 shows the comparison between the effects of SCC and rocuronium in terms of time from the start of administration of neuromuscular blocking agent to a T1 of zero. There was no significant difference between the groups.

Table 2 shows the time from commencement of administration of neuromuscular blocking agents to recovery of T1 to 10 and 90%, seizure duration, and time to first spontaneous breath in the two groups. The time to recovery of T1 to 90% in subjects treated with 16 mg/kg sugammadex was shorter than that in subjects treated with SCC ($p = 0.046$), and the time to recovery of T1 to both 10 and 90% in subjects treated with 4 mg/kg sugammadex was longer than that in subjects treated with SCC ($p < 0.01$). The time to first spontaneous breath in subjects treated with 16 mg/kg sugammadex was shorter than that in subjects treated with SCC ($p = 0.045$), and the time to first spontaneous breath in subjects treated with 4 mg/kg sugammadex was longer than that in subjects treated with SCC ($p < 0.01$). No significant difference in seizure duration was found among the four groups.

No adverse effects, such as nausea, vomiting, myalgia, or headache occurred with either relaxant. In addition, no symptoms of recurarization, for example respiratory depression (indicated by a decrease in SpO_2 less than 90% without supplementary oxygen supply) were seen in any of the patients treated with rocuronium–sugammadex (4, 8, or 16 mg/kg sugammadex) during the observation period of up to 12 h after the administration of rocuronium–sugammadex, when the patients were in the ward.

Table 1 Time from commencement of administration of neuromuscular blocking agents to a T1 of zero with each drug

	SCC	Rocuronium
Time to T1 of 0% (s)	109 ± 28	123 ± 28
p value	0.13	

Discussion

This study showed that:

1. the onset of action of 0.6 mg/kg rocuronium is equivalent to that of 1 mg/kg SCC for muscle relaxation during ECT; and
2. 8 mg/kg sugammadex is adequate for reversal of muscle relaxation induced by 0.6 mg/kg rocuronium during ECT.

Although Trollor and Sachdev [5] suggested the possible safety of the use of SCC in cases with neuroleptic malignant syndrome, SCC is thought to be a potent trigger for malignant hyperthermia (MH) [2]. Moreover, use of SCC is associated with a variety of adverse events and contraindications [2]. To avoid these, some researchers examined other neuromuscular agents, for example vecuronium [6, 7], mivacurium [8–10], rapacuronium [11] and rocuronium [12] during ECT. Kelly and Brull [10] demonstrated the safety of mivacurium as an alternative to SCC. In contrast, Cheam et al. [8] reported that a low dose of mivacurium was less effective than SCC. Another study of the safety of vecuronium reported that vecuronium shortened seizure duration and prolonged anesthetic time [6].

Rocuronium is potentially useful for muscle relaxation during ECT. However, before our previous study [4] there

Table 2 Time from commencement of administration of neuromuscular blocking agents to recovery of T1 to 10 and 90%, seizure duration, time to first spontaneous breath, and interval between rocuronium and sugammadex administration with each drug

	Recovery of T1 to 10% (s)	Recovery of T1 to 90% (s)	Time to first spontaneous breath (s)	Seizure duration (s)	Time from administration of rocuronium to administration of sugammadex (s)
SCC	310 ± 38	429 ± 65	273 ± 43	36 ± 6	
Sugammadex, 16 mg/kg	280 ± 54	387 ± 63*	233 ± 53*	38 ± 4	134 ± 7
Sugammadex, 8 mg/kg	324 ± 68	462 ± 66	267 ± 69	40 ± 7	132 ± 8
Sugammadex, 4 mg/kg	407 ± 74*,#	563 ± 45*,#	360 ± 59*,#	39 ± 5	134 ± 8

SCC, succinylcholine

* $p < 0.05$ compared with SCC

# $p < 0.05$ compared with sugammadex 16 mg/kg group

was only one report evaluating the effects of rocuronium versus SCC on clinical recovery from ECT [12]. Turkkal et al. [12] reported that although the time to first spontaneous breath was longer in the rocuronium group than in the SCC group, no significant differences were detected between the two groups in terms of eye opening, head lift, or tongue depressor testing. However, the dosage of rocuronium used in the study of Turkkal et al. [12] was relatively small (0.3 mg/kg), which is thought to be inadequate for muscular paralysis, because a dose of 0.3 mg/kg IV is rocuronium's ED_{50} dose for the laryngeal adductor muscles, this being half of the recommended intubating dose for rocuronium. Rocuronium (0.6–1.2 mg/kg) typically produces complete neuromuscular block within 2 min, compared with an average of 1 min with 1 mg/kg SCC [13]. High-dose rocuronium (1.0–1.2 mg/kg) has been recommended by some researchers as an effective alternative to SCC for rapid sequence induction. However, a meta-analysis of the Cochrane Review [14] concluded that intubation conditions do not statistically significantly differ with the administration of SCC and rocuronium when propofol is used to rapidly induce anesthesia. Indeed, in this study, no difference in the time from commencement of administration of neuromuscular blocking agents to T1 zero was found between the two groups. Hence, doses of 0.6 mg/kg rocuronium and 1 mg/kg SCC are appropriate for muscle relaxation during ECT.

Sluga et al. [15] compared tracheal intubation conditions with the use of 0.6 mg/kg rocuronium and 1 mg/kg SCC in emergency cases and showed that the time interval from injection of the neuromuscular blocking agents to the cessation of a visible motor response to continuous single-twitch nerve stimulation of the ulnar nerve was shorter in the SCC group (median time 40 s) than in the rocuronium group (median time 70 s). Although there was a tendency towards a longer interval between commencement of administration of neuromuscular blocking agents and T1 zero in the rocuronium group in this study, the difference between the two groups was not significant. The possible cause of this difference, as we previously showed [16], is that the onset of action of muscle relaxants is greatly affected by cardiac output before injection. Another possibility is that Sluga et al. [15] selected 1.5 mg/kg propofol with 2 μg/kg fentanyl for anesthetic induction. The difference in the anesthetic regime may also be responsible for the different results.

Lee et al. [3] compared the time required for sugammadex reversal of profound rocuronium-induced neuromuscular block with time to spontaneous recovery after SCC. In their study, either 1.2 mg/kg rocuronium or 1 mg/kg SCC was used for block of neuromuscular transmission and facilitation of tracheal intubation. Sugammadex (16 mg/kg) was administered 3 min after rocuronium administration. Mean times to recovery of T1 to 90% were significantly faster in the rocuronium–sugammadex group than in the SCC group. Hence, they concluded that reversal of profound high-dose rocuronium-induced neuromuscular block (1.2 mg/kg) with 16 mg/kg sugammadex was significantly faster than spontaneous recovery from 1 mg/kg SCC. In an earlier report from Gijsenbergh et al. [17] with an intubating dose of 0.6 mg/kg rocuronium, the TOF ratio returned to 0.9 within 2 min after administration of 8.0 mg/kg sugammadex given 3 min after the administration of rocuronium. Pühringer et al. [13] examined the dose-dependent effects of sugammadex for reversal of profound neuromuscular block. Sugammadex (2, 4, 8, 12, or 16 mg/kg) was administrated 3 min after the administration of 1.0 or 1.2 mg/kg rocuronium. The time to recovery of the TOF ratio to 0.9 with sugammadex was faster in a dose-dependent manner. These two reports showed that although the recovery speed of the TOF ratio to 0.9 with sugammadex was dose-dependent, its efficacy was unchanged. Our study showed that although 16 mg/kg sugammadex resulted in the fastest recovery of the TOF ratio to 0.9 in the case of 0.6 mg/kg rocuronium, 8 mg/kg sugammadex had equipotent effects on the recovery of the TOF ratio to 0.9 compared with the use of SCC during ECT.

Batistaki et al. [18] reported successful anesthetic management of a patient with pseudocholinesterase deficiency by use of rocuronium reversed by sugammadex in a series of ECT sessions. In a preliminary report, we showed the potential efficacy of the use of rocuronium–sugammadex as a muscle relaxant during ECT [4]. Hence, rocuronium–sugammadex could also be useful for muscle relaxation during ECT for patients for whom the use of SCC is contraindicated. Our report implies that a combination of rocuronium–sugammadex, using 0.6 mg/kg rocuronium, may be adequate for inducing muscle paralysis during ECT. In addition, 8 mg/kg sugammadex produced adequate recovery from the muscular relaxation induced by 0.6 mg/kg rocuronium during ECT.

In this study, the patients were administered a combination of rocuronium–sugammadex repeatedly once a day for a week during the study. Although this repeated administration may possibly have adverse effects on the patients, we did not find any adverse effects (nausea, vomiting, prolongation of the QTc interval), and more specifically, recurarization, with any of the three doses of sugammadex. Batistaki et al. [18] reported that a combination of rocuronium–sugammadex used every 48 h for 8 consecutive ECT sessions proved to be effective and safe in a situation where SCC was contraindicated. Our study also confirms the potential usefulness of rocuronium–sugammadex for muscle relaxation during ECT for patients for whom the use of SCC is contraindicated, for example those with severe osteoporosis, amyotrophic lateral sclerosis, and a history of neuroleptic syndrome.

Another consideration is that the combination of rocuronium–sugammadex is eliminated by the kidney, so that it is possible that its elimination could be prolonged in patients with impaired renal function, which could induce adverse effects in these patients. All patients included in this study had normal renal function, as shown by normal serum creatinine and BUN levels. However, care should be taken when using sugammadex for patients with impaired renal function. Use of the combination of rocuronium–sugammadex would also be disadvantageous compared with SCC in cases where re-use of rocuronium is required immediately after administration of sugammadex.

In conclusion, we demonstrated the efficacy of rocuronium–sugammadex as an alternative to SCC for muscle relaxation during ECT and showed that 8 mg/kg SG has equipotent recovery time from muscular relaxation compared with 1 mg/kg SCC during ECT.

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Conflict of interest No authors have any conflicts of interest in association with this article.

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Dexmedetomidine decreases hyperalgesia in neuropathic pain by increasing acetylcholine in the spinal cord

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HIGHLIGHTS

- Interaction between α2-adrenergic and acetylcholine in neuropathic pain was examined.
- Spinal α2-adrenoceptor activation suppressed hyperalgesia in rats with nerve injury.
- The anti-hyperalgesia was reversed by an intrathecal muscarinic receptor antagonist.
- Spinal α2-adrenoceptor activation increased acetylcholine in rats with nerve injury.
- Acetylcholine contributes to analgesia from spinal α2-adrenoceptor activation.

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ABSTRACT

The activation of α2-adrenoceptors has attracted attention as a therapeutic target for neuropathic pain, which remains a clinical challenge. In the present study, we examined the interaction between α2-adrenergic and cholinergic signaling in a rat model of neuropathic pain induced by spinal nerve ligation (SNL). Intrathecal administration of dexmedetomidine, which is a selective α2-adrenoceptor agonist (0.1–1.0 μg), dose-dependently suppressed hyperalgesia in SNL rats but did not alter paw withdrawal thresholds in normal rats. The analgesic effect of dexmedetomidine was abolished by intrathecal pre-treatment with idazoxan (30 μg) and atropine (30 μg), which antagonize the α2-adrenoreceptor and muscarinic receptor, respectively. In vivo microdialysis in the lumbar spinal dorsal horn revealed that acetylcholine concentrations increased after dexmedetomidine perfusion (1 μM), but only in SNL rats. The combination of an ineffective dose of intrathecal dexmedetomidine with intraperitoneal donepezil, which is a cholinesterase inhibitor, decreased neuropathic hypersensitivity. These results suggest that plasticity of the spinal noradrenergic–cholinergic axis only occurs in neuropathic pain states. Thus, drug combinations that strengthen the noradrenergic–cholinergic interaction may provide therapeutic benefit in neuropathic pain.

1. Introduction

Peripheral nerve injury can result in neuropathic pain, which is associated with various changes in sensory processing from primary afferent neurons to the spinal cord and supraspinal regions. Only a few drugs, including calcium channel α2–δ ligands and antidepressants, have been approved to treat neuropathic pain [4]. These drugs share a common pharmacological mechanism that involves the activation of spinal α2-adrenoceptors [9,10,19], which play an important role in the suppression of neuropathic pain. Previous studies suggest that intrathecal injection of the α2-adrenoceptor agonist clonidine has increased potency and efficacy against neuropathic pain states in animals [20] and humans [5]. This effect is thought to be mediated through the downstream activation of inhibitory cholinergic interneurons; thus, α2-adrenoceptor agonists, which normally inhibit spinal cholinergic interneurons, excite them after nerve injury, and the resulting acetylcholine (ACh) release is thought to be critical for the analgesic effects of spinal α2-adrenoceptor activation [8,18]. However, in vivo studies to date have not directly measured ACh levels in the spinal cord after the administration of α2-adrenoceptor agonists in neuropathic pain states.

The purpose of the current study was to test whether the analgesic effects of spinal α2-adrenoceptor activation in neuropathic pain states are mediated through plasticity of the noradrenergic–cholinergic axis by behavioral studies and directly measuring ACh release in the lumbar spinal cord using in vivo microdialysis.

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2. Material and methods

2.1. Surgical preparation

The study was approved by the Animal Care and Use Committee of the Gunma University School of Medicine (Maebashi, Japan). Male Sprague-Dawley rats (250 g) were used in all experiments. The animals were housed under a 12-h light–dark cycle, with food and water available ad libitum. As previously described [12], the animals were anesthetized with inhaled isoflurane in oxygen, and the right L5 spinal nerve was tightly ligated with 5-0 silk and cut just distal to the ligature. The wound was closed and the animals were allowed to recover for 2 weeks.

2.2. Behavioral testing

Withdrawal threshold to pressure applied to the hind paw, expressed in grams, was measured using an analgesimeter (37215 Ugo Basile, Comerio, Italy) as previously described [21]. The device applies increasing pressure to the hind paw. When the animal withdraws the paw, the pressure is immediately released, and the nociceptive threshold is measured in grams. A cutoff of 250 g was used to prevent potential tissue injury. The experimenter was blinded to the treatment group. All animals were trained for 3 days with the device before baseline values were recorded.

2.3. Drugs and their administration

Drug testing was performed 2–3 weeks after nerve ligation. The rats were intrathecally injected with dexmedetomidine, which is a selective α2-adrenoceptor agonist (0.1, 0.3, and 1.0 μg) or intraperitoneally injected with donepezil, which is a central nervous system-penetrating cholinesterase inhibitor (0.3, 0.6, and 1.0 mg/kg). Antagonist studies against dexmedetomidine-mediated analgesia were performed using idazoxan (30 μg) and atropine (30 μg), which antagonize α2-adrenoceptors and muscarinic receptors, respectively. Saline or each antagonist was administered intrathecally 15 min prior to dexmedetomidine injection. The doses of the antagonists were selected according to previous studies [2,19] and our preliminary studies. For the combination drug study, dexmedetomidine (0.1 μg, intrathecal) and donepezil (0.3 mg/kg, intraperitoneal) were administered simultaneously. For intrathecal administration, dexmedetomidine, idazoxan, and atropine were dissolved in 5 μl of saline and injected at the L5–6 intervertebral space using a 30-gauge needle. For intraperitoneal administration, donepezil was dissolved in 0.5 ml of saline. Dexmedetomidine was a gift from Orion Pharma (Espoo, Finland). Donepezil was a gift from Eisai Co. (Tokyo, Japan). Other drugs were purchased from Sigma Co. (St. Louis, MO).

2.4. Microdialysis studies

Microdialysis studies were performed to measure ACh levels after perfusion of dexmedetomidine in the spinal dorsal horn in normal rats or SNL rats (2–3 weeks after SNL) according to the method of a previous study [17]. Anesthesia was induced by intraperitoneal injection with urethane (1.2–1.5 g/kg) and maintained with 0.5% isoflurane in 100% oxygen through a nose cone. The left femoral vein was cannulated for transfusion of saline at a rate of 1 ml/h. The rectal temperature was maintained at 37–38 °C by a heating pad placed beneath the animal. The L3–5 level of the spinal cord was exposed by a thoraco-lumbar laminectomy, and the rat was placed in a stereotaxic apparatus. The microdialysis probe was inserted from just lateral to the dorsal root and advanced at a 30° angle to a depth of 2 mm using a micromanipulator (model WR-88, Narishige, Japan). The microdialysis probe comprised a 2-mm length of cylinder-shaped dialysis membrane (OD = 0.22 mm, ID = 0.20 mm, AI-8-02 Eicom Co., Kyoto, Japan). The microdialysis probe was perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$) at a constant flow rate (1 μl/min) using a syringe pump (ESP-64, Eicom Co.). After 120 min of constant perfusion, two consecutive samples were collected to determine basal ACh concentrations in the dialysate. For ACh measurements with dexmedetomidine, Ringer's solution (control) or dexmedetomidine (1 μM) with Ringer's solution was perfused and 30-min perfusate fractions were collected.

Samples (30 μl) were automatically injected and the ACh concentration was analyzed using high-performance liquid chromatography with electrochemical detection by an HTEC-500 analyzing system (Eicom Co.). The chromatographic conditions were as follows: the mobile phase consisted of 50 mM $KHCO_3$ containing 300 mg/l sodium 1-decanesulfonate (pH 8.5) and 50 mg/l EDTA-2Na. The column was a EICOMPAC AC-GEL (2.0 mm × 150 mm; Eicom Co.). The working electrode was glassy carbon (WE-3G, Eicom Co.) with a flow rate of 0.15 ml/min. The detector voltage was set at 0.45 V. The detector temperature was set at 33.0 °C. The retention time for ACh was 12.4 min. The detection limit of this assay was 10 fg per injection (information from Eicom Co.).

2.5. Statistics

Data from the behavioral and microdialysis studies were normally distributed and are presented as the mean ± SEM. The time-course data were analyzed using a two-way analysis of variance (ANOVA). Student–Neuman–Keuls post hoc tests were performed for between-group comparisons at each time point. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Anti-hyperalgesic effect of dexmedetomidine

Intrathecal administration of dexmedetomidine (0.1–1.0 μg) produced dose-dependent anti-hyperalgesia in SNL rats ($P < 0.05$ by two-way ANOVA; Fig. 1). The peak effect was observed

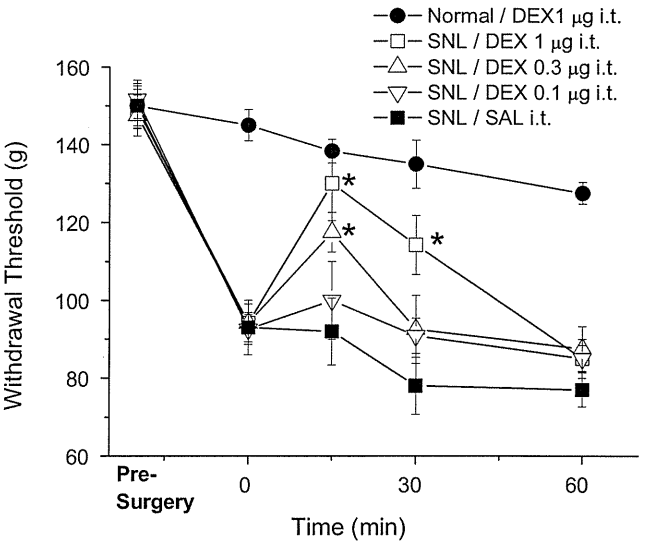

Fig. 1. Effect of intrathecal administration of dexmedetomidine (DEX) on the withdrawal threshold to pressure applied to the hind paw in normal and SNL rats. Withdrawal thresholds are expressed as the mean ± SEM for 6 rats in each group. *$P < 0.05$ compared with the saline (SAL)-treated group at each time point (Student–Neuman–Keuls post hoc test after two-way ANOVA).

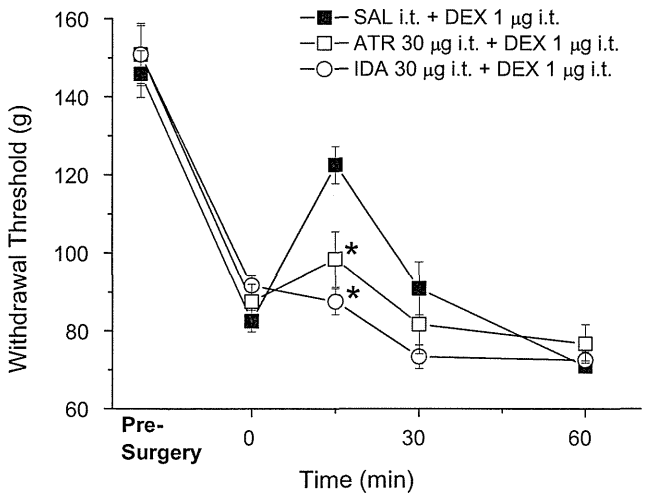

Fig. 2. Effect of pretreatment with idazoxan (IDA) and atropine (ATR), which antagonize the α2-adrenoceptor and muscarinic acetylcholine receptor, respectively, on the anti-hyperalgesic effect of dexmedetomidine (DEX; 1 μg). Saline (SAL) or 30 μg of each antagonist was administered intrathecally 15 min prior to intrathecal injection of dexmedetomidine. Withdrawal thresholds are expressed as the mean ± SEM for 6 rats in each group. *$P < 0.05$ compared with the saline-treated group at each time point (Student–Neuman–Keuls post hoc test after two-way ANOVA).

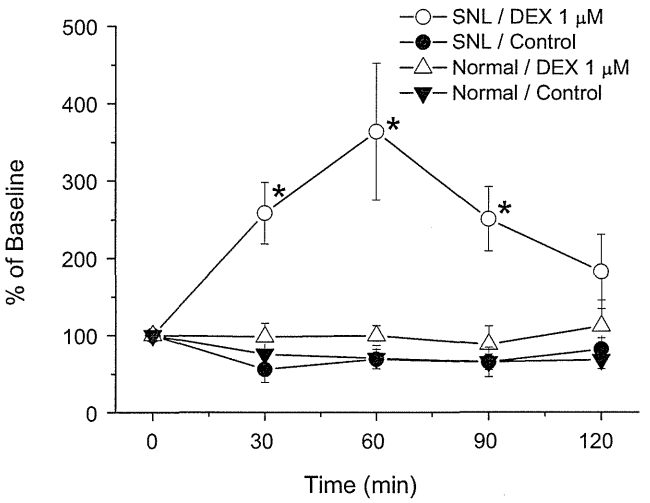

Fig. 3. Microdialysis to detect increased spinal acetylcholine levels. Normal rats ($n = 6$) or SNL rats ($n = 6$) were perfused with dexmedetomidine (DEX) solution (1 μM) or Ringer's solution (control). Data are presented over time as a percentage of the baseline. *$P < 0.05$ compared with the control (SNL) group at each time point (Student–Neuman–Keuls post hoc test after two-way ANOVA).

15 min after administration of 1.0 μg dexmedetomidine, and the effect continued for 30 min after administration when compared with the saline-treated group ($P < 0.05$ by Student–Neuman–Keuls post hoc test). Intrathecal administration of the highest dose of dexmedetomidine did not affect the withdrawal threshold in normal, i.e., uninjured, rats. Adverse behavioral effects, such as motor effects, sedation, or agitation, were not observed. Intrathecal pretreatment with atropine (30 μg) or idazoxan (30 μg) attenuated the anti-hyperalgesic effect of dexmedetomidine ($P < 0.05$ by two-way ANOVA; Fig. 2).

3.2. ACh increase in the spinal cord after dexmedetomidine administration

In the lumbar spinal dorsal horn, the baseline ACh concentration before the infusion was not different between normal rats and SNL rats (2.32 ± 0.41 pg/30 μl in normal rats and 2.11 ± 0.43 pg/30 μl in SNL rats). In the control group perfused with Ringer's solution, the ACh concentration in the dialysate did not change over time. In the group perfused with dexmedetomidine (1 μM), the ACh concentration increased within 30 min and reached approximately 350% of the baseline value in SNL rats at 60 min ($P < 0.05$ by Student–Neuman–Keuls post hoc test after two-way ANOVA). In contrast, the same concentration of dexmedetomidine did not affect the concentration of ACh in normal rats (Fig. 3).

3.3. Synergistic anti-hyperalgesic effect of dexmedetomidine and donepezil

To evaluate the analgesic interaction between spinal α2-adrenoreceptor activation and cholinergic signaling in hyperalgesia after SNL, donepezil was used to increase the ACh level. Intraperitoneal administration of donepezil (0.3–1.0 mg/kg) produced a dose-dependent anti-hyperalgesic effect in SNL rats ($P < 0.05$ by two-way ANOVA; Fig. 4A). The peak effect was observed 15 min after the administration of 1.0 mg/kg donepezil, and the effect continued for over 60 min when compared with the saline-treated group ($P < 0.05$ by Student–Neuman–Keuls post hoc test) without any adverse effect. The combination of intrathecal dexmedetomidine (0.1 μg) and intraperitoneal donepezil (0.3 mg/kg) at doses that individually failed to affect the withdrawal threshold produced anti-hyperalgesia when compared with the agents administered individually or with saline administration ($P < 0.05$ by two-way ANOVA; Fig. 4B).

4. Discussion

The activation of spinal α2-adrenoceptors directly inhibits nociceptive neurotransmission by reducing the release of neurotransmitters such as substance P and glutamate from primary afferent terminals [16] and by hyperpolarizing dorsal horn neurons through the G-protein-mediated activation of potassium channels [22]. Consistent with previous studies that demonstrated the efficacy of the intrathecal α2-adrenoceptor agonist clonidine in neuropathic pain [5,20], we demonstrated that the intrathecal administration of dexmedetomidine produced a dose-dependent anti-hyperalgesic effect in SNL rats. In contrast, dexmedetomidine did not affect the withdrawal threshold in normal rats. Although several potential causes for the difference in effect between animals with and without nerve injury have been proposed, including increased expression of inhibitory α2-adrenoceptors on afferent neurons expressing calcitonin gene-related peptide [6] and increased G protein-coupling efficiency in spinal α2-adrenoceptors [1], the activation of inhibitory cholinergic interneurons by α2-adrenoceptors may be the most important mechanism [8,18].

Previous studies demonstrated such interactions between spinal α2-adrenoceptors and cholinergic interneurons. For example, intrathecally administered clonidine increased the concentration of ACh in the cerebrospinal fluid [3] and microdialysates from the spinal cord dorsal horn [13] in sheep. Moreover, we previously demonstrated that α2-adrenoceptor agonists such as clonidine and dexmedetomidine facilitate KCl-evoked ACh release from lumbar dorsal horn synaptosomes in SNL rats [18]. Hayashida et al. demonstrated that dexmedetomidine inhibited and excited KCL-evoked ACh release in the synaptosomes of normal and SNL rats, respectively, and this change depended on a shift from direct inhibition via the Gi/o protein to direct excitation via the Gs protein [8]. Consistent with these synaptosomal experiments, we demonstrated that dexmedetomidine increased the ACh level in the lumbar spinal cord of SNL rats, but not normal rats, using in vivo microdialysis. Furthermore, the anti-hyperalgesic effect of dexmedetomidine

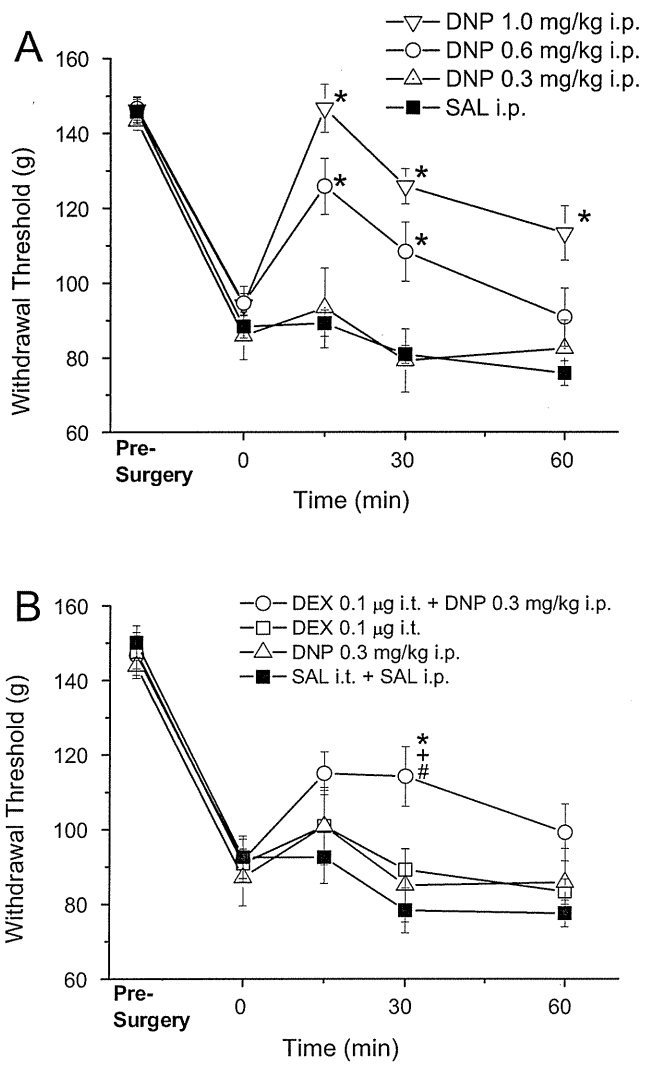

Fig. 4. Effect of intraperitoneal administration of donepezil (DNP) on the withdrawal threshold to pressure applied to the hind paw in SNL rats (A). Withdrawal thresholds are expressed as the mean ± SEM for 6 rats in each group. $^{*}P < 0.05$ compared with saline (SAL)-treated group at each time point (Student–Neuman–Keuls post hoc test after two-way ANOVA). Effect of the combination of intrathecal dexmedetomidine (DEX) and intraperitoneal donepezil (DNP) on the withdrawal threshold to pressure applied to the hind paw in rats subjected to spinal nerve ligation (B). Withdrawal thresholds are expressed as the mean ± SEM for 6 rats in each group. $^{*}P < 0.05$ compared with the saline (SAL)-treated group, $^{\#}P < 0.05$ compared with the dexmedetomidine-treated group, $^{+}P < 0.05$ compared with the donepezil treated-group at each time point (Student–Neuman–Keuls post hoc test after two-way ANOVA).

was prevented by intrathecal pretreatment with atropine or idazoxan. These results suggest that the ACh increase in the spinal cord is crucial for the anti-hyperalgesic effect from spinal α2-adrenoceptor activation in SNL rats. A recent study demonstrated that dorsal horn cholinergic interneurons are the main source of ACh in the dorsal horn [15]. Therefore, the infused dexmedetomidine may have directly affected to the cholinergic interneurons in our microdialysis studies. The peak anti-hyperalgesic effect of dexmedetomidine was observed 15 min after intrathecal administration, whereas the peak ACh increase occurred 60 min after the initiation of dexmedetomidine perfusion (second fraction of the dialysates). This temporal difference may be derived from differences in the drug application and measurement methods between the behavioral and microdialysis studies.

Cholinergic agents exert anti-nociceptive effects in animals [23], and the intrathecal administration of cholinesterase inhibitors such as neostigmine and edrophonium produces analgesia in SNL rats [11,14]. In a recent study, the oral administration of donepezil, which a cholinesterase inhibitor that is approved for the symptomatic treatment of Alzheimer's dementia, produced an anti-allodynic effect in SNL rats [2]. In the present study, intraperitoneal administration of donepezil also produced dose-dependent anti-hyperalgesia in SNL rats. Furthermore, the combination of individually ineffective doses of intrathecal dexmedetomidine (0.1 μg) and intraperitoneal donepezil (0.3 mg/kg) produced anti-hyperalgesia. Several antidepressants (e.g., tricyclic antidepressants as well as serotonin and noradrenaline reuptake inhibitors) and calcium channel α2-δ ligands (e.g., gabapentin and pregabalin) that are routinely used in the treatment of neuropathic pain [4] rely on spinal α2-adrenoceptors as well as cholinergic mechanisms to provide analgesia [10,19]. The current study provides further support for clinical trials to examine the supplementation of these drugs with donepezil in therapeutic regimens for neuropathic pain [7].

5. Conclusions

In the spinal nerve ligation model of neuropathic pain, analgesia mediated by the activation of α2-adrenoceptors is augmented by increased ACh in the lumbar spinal cord. These results further our understanding regarding the spinal mechanisms by which activation of α2-adrenoceptors produce analgesia in neuropathic states and strengthen the rationale for their clinical application of cholinergic agents.

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