Supplemental Material

Sustained increase in α5GABA$_A$ receptor function impairs memory after anesthesia

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Experimental animals. Gabra5$^{-/-}$ mice and wild-type mice (C57BL/6J × SvEv129) were housed in the Animal Care Facility, University of Toronto. The generation, genotyping, and characterization of *Gabra5*$^{-/-}$ mice have been previously described (1). For all behavioral tests, age-matched 3- to 5-month-old male wild-type and *Gabra*5$^{-/-}$ mice were used. For electrophysiological and cell-surface biotinylation experiments, 1- to 4-month-old male mice were studied. Researchers were blinded to all drug conditions. In addition, researchers were blinded to the genotype for all behavioral experiments.

Anesthesia. Mice were treated with sedating doses of etomidate (8 mg/kg, i.p.), dexmedetomidine (200 μg/kg, i.p.) or isoflurane (0.7%, 20 min). Physiological saline (i.p.) was

used as the vehicle for etomidate and dexmedetomidine, and 30% O_2 (20 min) mixed with air was used as the vehicle for isoflurane. Anesthetizing doses of etomidate (20 mg/kg, i.p.) or isoflurane (1.3%, 1h) and their corresponding vehicles (propylene glycol 26% v/v in physiological saline and 30% O_2, respectively) were also used. The sedating and anesthetizing doses of etomidate and isoflurane were selected from the literature to approximate the ED_{50} and ED_{100} for the loss of righting reflex, respectively (2-4).

To prevent hypoxia during anesthesia, each mouse was placed in an air-tight acrylic chamber (27 cm × 10 cm × 10 cm) that was flushed with supplemental oxygen and medical air (70% air, 30% O_2) delivered at a flow of 1 L/min. The concentrations of O_2, CO_2 (and isoflurane when applicable) in the chamber were continuously monitored with a commercial gas analyzer (Datex Ohmeda, Mississauga, Ontario, Canada). In a subset of mice, transcutaneous oxygen saturation was measured at a frequency of 15 Hz with a mouse pulse oximetry sensor (MouseOx, Starr Life Sciences Corp., Allison PA) that was placed on a shaved area of the throat over the carotid arteries. None of the anesthetic treatments caused hypoxia as oxygen saturation remained above 98% during etomidate (20 mg/kg i.p.) and isoflurane (1.3%, 1 h). To prevent hypothermia, the temperature of the chamber was maintained at 35°C with a heating blanket (5). Mice were not tested for immobility with the tail pinch assay or for LORR during anesthesia to avoid unnecessary stimulation.

Novel object recognition memory assay. Mice were handled for at least 10 min daily for 5 days before the start of behavioral experiments. Object recognition was assessed in a 20 cm × 20 cm × 30 cm opaque chamber in a dimly lit room. Each mouse was habituated to the chamber for 15 min 24 h before testing. During the training phase, the mouse was allowed to explore two

identical “sample” objects for 10 min. The mouse was then returned to its home cage for a retention period of 1 h. The mouse was reintroduced to the training context and presented with one familiar, sample object and one novel object for 5 min. Movement and interaction with the objects was recorded with a video camera that was mounted above the chamber and exploratory behavior was measured by a blinded observer. Exploratory behavior was defined as sniffing, licking, or touching the object while facing the object. Memory was assessed by measuring the discrimination ratio (i.e. the ratio of time spent exploring the novel object to the time spent exploring both objects). Mice that exhibited a discrimination ratio greater than the value of chance (0.5) were deemed to have remembered the familiar object. Animals that did not interact for a minimum of 1 s with each object during the test period were excluded. Total interaction time with both objects was compared between groups to determine whether the treatments affected locomotor activity or exploration during the testing phase. Each mouse treated with dexmedetomidine, etomidate (8 mg/kg, i.p.) or the corresponding vehicle was trained and tested 3 times; 24 h, 72 h, and 1 week after anesthetic exposure. Each mouse treated with etomidate (20 mg/kg, i.p.) was trained and tested at 4 times; 24 h, 72 h, 1 week and 2 weeks. Different pairs of objects were used for each training session. A separate group of mice was treated with the inverse agonist for α5GABA$_A$Rs, L-655,708 (0.5 mg/kg i.p.) or vehicle (2% DMSO I physiological saline) 30 min before training on the object recognition task.

Electrophysiology in hippocampal slices. After live decapitation, brains were removed and placed in ice-cold, oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) that contained (in mM): 124 NaCl, 3 KCl, 1.3 $MgCl_2$, 2.6 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 D-glucose with the solution osmolarity adjusted to 300–310 mOsm. Coronal brain slices (350

μm) were prepared with a VT1200S vibratome (Leica, Deerfield, Illinois). The slices were allowed to recover for at least 1 h at room temperature (23–25 °C) before being transferred to a submersion recording chamber, where they were perfused with ACSF at 3–4 ml/min. All recordings were performed at room temperature using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, California) controlled with pClamp 9.0 software via a Digidata 1322A interface (Molecular Devices, Sunnyvale, California).

For extracellular recording of field postsynaptic potentials (fPSPs), the pipettes were filled with ACSF and placed in the stratum radiatum of Cornus Ammonis 1 (CA1). The Schaffer collateral pathway was stimulated with 0.1 ms pulses delivered by a concentric bipolar tungsten electrode (Rhodes Medical Instruments, Summerland, California). To record plasticity of fPSPs, the slope of fPSPs was measured at baseline for at least 10 min with a stimulation frequency of 0.05 Hz and using a stimulation intensity that produced a half-maximal response. Slices were then stimulated at 20 Hz (600 pulses), and fPSPs were monitored for 60 min after stimulation. The 20 Hz stimulation protocol was selected because the activity of extrasynaptic α5GABA$_A$ receptors modifies the plasticity of fPSPs under these experimental conditions (6). The average of the last 5 min of recording was compared with the average of the baseline fPSPs. Post-tetanic depression was measured during the first 2 min after stimulation. Short-term depression was measured during the first 15 min after stimulation. Paired pulse facilitation was measured during the 20 Hz stimulation (inter-stimulus interval of 50 ms). The slope of the fPSP after the first stimulus pulse (fPSP1) and the second stimulus pulse (fPSP2) were measured. The paired pulse ratio was calculated by dividing the slope of fPSP2 by the slope of fPSP1.

For whole-cell voltage-clamp recordings, the pipettes (2–3 MΩ) were filled with the intracellular solution containing (in mM): 140 CsCl, 10 HEPES, 11 EGTA, 4 Mg_2ATP, 2 $MgCl_2$,

1 $CaCl_2$, 2 TEA (pH 7.3 with CsOH, 290–300 mOsm). Currents were sampled at 10 kHz. All cells were recorded at a holding potential of −60 mV and automatic capacitance compensation was applied. To measure the tonic current, the $GABA_A$ receptor competitive antagonist, bicuculline (10 μM) or L-655,708 (200 nM), was applied. The tonic current was quantified by measuring the change in the holding current from segments containing no synaptic events. Exogenous GABA (5 μM) was added to ACSF. The addition of GABA (5 μM) to the perfusate will result in GABA concentrations similar to physiological levels of extracellular GABA (0.2–0.25 μM) measured *in vivo* with microdialysis because of active reuptake of GABA in brain slices (7).

Primary cell culture. Primary cultures of hippocampal neurons were prepared from Swiss Webster mice (Charles River, Montreal, Canada), as described previously (8). Briefly, fetal pups (embryonic day 18) were removed from mice euthanized by cervical dislocation. The hippocampi were dissected from each fetus and placed in an ice-cooled culture dish. Neurons were then dissociated by mechanical titration using two Pasteur pipettes (tip diameter, 150– 200 μm) and plated on 35-mm culture dishes at a density of approximately 1×10^6 cells/ml. The culture dishes were coated with collagen or poly-D-lysine (Sigma-Aldrich Co., St. Louis, Missouri). For the first 5 days *in vitro*, cells were maintained in minimal essential media (MEM) supplemented with 10% fetal bovine serum and 10% horse serum (Life Technologies, Grand Island, New York). The neurons were cultured at 37 ^oC in a 5% CO_2-95% air environment. After the cells had grown to confluence, 0.1 ml of a mixture of 4 mg 5-fluorodeoxyuridine and 10 mg uridine in 20 ml MEM was added to the extracellular solution to reduce the number of dividing cells. Subsequently, the media was supplemented with 10% horse serum and changed every 3 or

4 days. Neurons were maintained in culture for 14 to 21 d prior to recording. To prepare microglia-neuron cocultures, cortical microglia were isolated from embryonic mice as described previously (9). Cells were maintained as described above and media was changed every 3 to 4 days. Once cell confluence was achieved, microglia were separated from the mixed glial cultures by gently shaking the dishes (200 rpm for 2 hr at 37° C) then centrifuging the supernatant. The pellet was suspended in neurobasal media and applied directly over cultured hippocampal neurons that had been grown for 10 to 14 days *in vitro*. For astrocyte culture, cortical astrocytes were isolated from embryonic day 18 (E18) mouse embryos as described previously (10). Cells were allowed to grow to confluence in MEM and 10% fetal bovine serum (FBS; Life Technologies, Grand Island, New York) for 14 d. Cells were then enzymatically dissociated with trypsin–EDTA (0.05%; Life Technologies, Grand Island, New York), and passaged three times to obtain a nearly pure astrocytic culture. Astrocytes were then plated at a density of 25,000 cells per dish. For astrocyte-neuron coculture, astrocyte cell suspension was placed over hippocampal neurons cultured at 14 d in neurobasal media. Astrocytes were monitored visually to ensure survival and confluence for the duration of the experiment. For all reported results, data were acquired from cells from at least three different dissections.

Whole-cell voltage-clamp recordings in cell culture. Whole-cell recordings were performed as described above with several exceptions. The extracellular solution contained the following (in mM): 140 NaCl, 2.0 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 25 HEPES, and 28 glucose (pH 7.4, 320–330 mOsm). Etomidate (0.25 μM or 1 μM) or vehicle solution was used to treat the culture dish for 1 h. The concentrations of 0.25 μM and 1 μM of etomidate were selected because they correspond to low, sedative and anesthetizing doses of etomidate, respectively (11, 12). The media was then

removed and replaced with fresh culture media. Recordings were performed 24 h later. To prepare conditioned supernatant media, astrocyte cultures were treated with etomidate (1 μM) for 1 h. The media was then removed and replaced with fresh culture media. The astrocytes were kept in the fresh media for 2 h before the conditioned media was collected and applied to neuronal culture for 24 h. To measure the amplitude of the tonic current, exogenous GABA (0.5 μM) was added to the extracellular solution and the change in holding current was measured during application of bicuculline (20 μM). GABA (0.5 μM) is similar to physiological levels of extracellular GABA that occur *in vivo (7).*

Cell-surface biotinylation. Coronal hippocampal slices (350 μm) were prepared 24 h, 1 week or 2 weeks after *in vivo* treatment with etomidate or isoflurane and placed in oxygenated ACSF (95% O_2, 5% CO_2) for 1 h to recover. Slices were then placed on ice and incubated twice with 0.75 mg/ml NHS-SS-biotin (Thermo Scientific, Rockford, Illinois) dissolved in DPBS (Gibco, Burlington, Ontario, Canada) for 30 min each time. Excess biotin was quenched and removed by washing slices 6 times with ice cold modified TBS containing (in mM): 25 Tris-Cl, 137 NaCl, 1 KCl, 2.3 $CaCl_2$, pH 7.4. Slices were then placed in lysis buffer (pH 7.4) containing complete protease inhibitor cocktail (Roche, Laval, Quebec, Canada) for homogenization. Insoluble material was removed by centrifugation. Bicinchoninic acid assay (Bio-Rad, Hercules, California) was performed to determine protein concentration. The supernatant lysates were incubated with Hi-Capacity NeutrAvidin beads (Thermo Scientific, Rockford, Illinois) for 16-18 h at 4°C. The beads were washed with PBS containing 0.05% SDS. Bound material was eluted with elution buffer containing (in mM): 50 Tris-Cl, 2% SDS, 2 DTT; protein concentration was determined using DC™ Protein Assay (Bio-Rad, Hercules, California) and subjected to SDS-

PAGE analysis. A western blot analysis with anti-$GABA_A$ receptor antibodies for α1 (Abcam, Cambridge, Massachusetts or Millipore, Billerica, Massachusetts), α5 (PhosphoSolutions, Aurora, Colorado), β3 (Thermo Scientific, Rockford, Illinois), or δ (Millipore, Billerica, Massachusetts) was performed. Anti-β-actin antibody (Millipore, Billerica, Massachusetts) and anti-Na^+/K^+ ATPase antibody (Developmental Studies Hybridoma Bank, Iowa City, Iowa) were also used. Blots were imaged using the Chemidoc XRS+ system (Bio-Rad, Hercules, California) and quantified using Image Lab software (Bio-Rad, Hercules, California). All samples were loaded onto two replicate blots in order to control for transfer errors. Receptor bands were normalized to their respective loading controls, which were Na^+/K^+ ATPase for surface protein and β-actin for total protein. Blots containing surface protein were probed for β-actin to determine the purity of isolated biotinylated surface protein. Data in each experiment are presented as a percentage of the mean of the control group.

Supplemental References

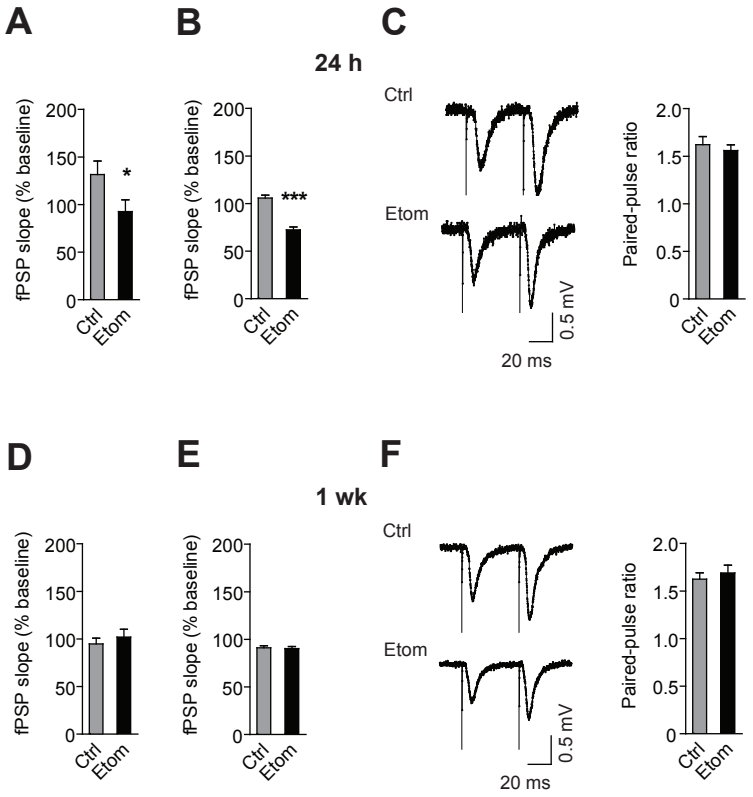
1. Collinson N, et al. Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the α5 subunit of the $GABA_A$ receptor. *J Neurosci.*2002;22(13):5572-5580.
2. Cheng VY, et al. α5$GABA_A$ receptors mediate the amnestic but not sedative-hypnotic effects of the general anesthetic etomidate. *J Neurosci.*2006;26(14):3713-3720.
3. Sonner JM, Gong D, Eger EI. Naturally occurring variability in anesthetic potency among inbred mouse strains. *Anesth Analg.*2000;91(3):720-726.
4. Sonner JM, et al. Effect of isoflurane and other potent inhaled anesthetics on minimum alveolar concentration, learning, and the righting reflex in mice engineered to express α1 γ-aminobutyric acid type A receptors unresponsive to isoflurane. *Anesthesiology.*2007;106(1):107-113.
5. Saab BJ, et al. Short-term memory impairment after isoflurane in mice is prevented by the α5 γ-aminobutyric acid type A receptor inverse agonist L-655,708. *Anesthesiology.*2010;113(5):1061-1071.
6. Martin LJ, et al. α5$GABA_A$ receptor activity sets the threshold for long-term potentiation and constrains hippocampus-dependent memory. *J Neurosci.*2010;30(15):5269-5282.
7. Bright DP, Smart TG. Methods for recording and measuring tonic $GABA_A$ receptor-mediated inhibition. *Front Neural Circuits.*2013;7.
8. MacDonald JF, Mody I, Salter MW. Regulation of N-methyl-D-aspartate receptors revealed by intracellular dialysis of murine neurones in culture. *J Physiol.*1989;414:17-34:17-34.

9. Deierborg T. Preparation of primary microglia cultures from postnatal mouse and rat brains. *Methods Mol Biol.*2013;1041:25-31.

10. Albuquerque C, Joseph DJ, Choudhury P, MacDermott AB. Dissection, plating, and maintenance of cortical astrocyte cultures. *Cold Spring Harb Protoc.*2009;(8):pdb.prot5273.

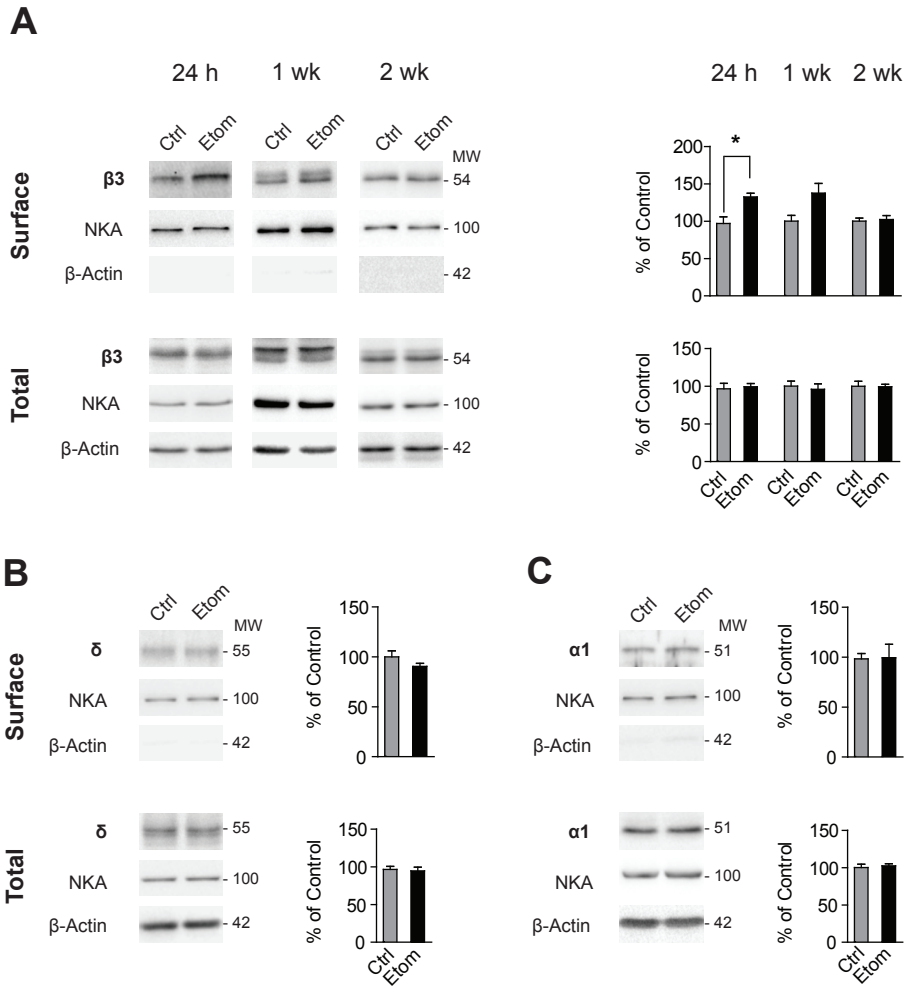
11. Benkwitz C, et al. Determination of the EC_{50} amnesic concentration of etomidate and its diffusion profile in brain tissue: Implications for *in vitro* studies. *Anesthesiology.*2007;106(1):114-123.

12. Alkire MT, Gorski LA. Relative amnesic potency of five inhalational anesthetics follows the Meyer-Overton rule. *Anesthesiology.*2004;101(2):417-429.

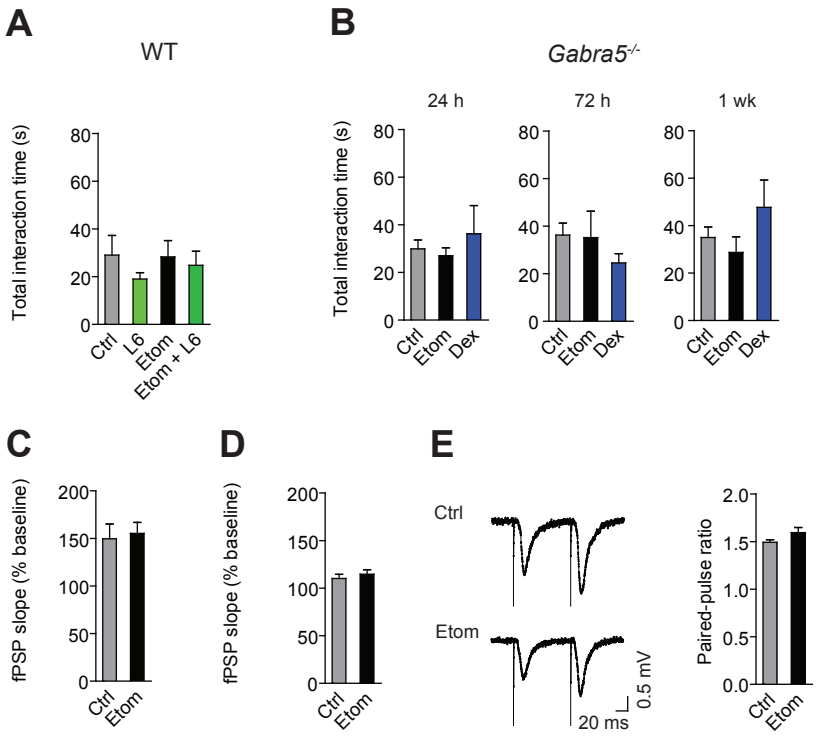


Supplementary Figure 1

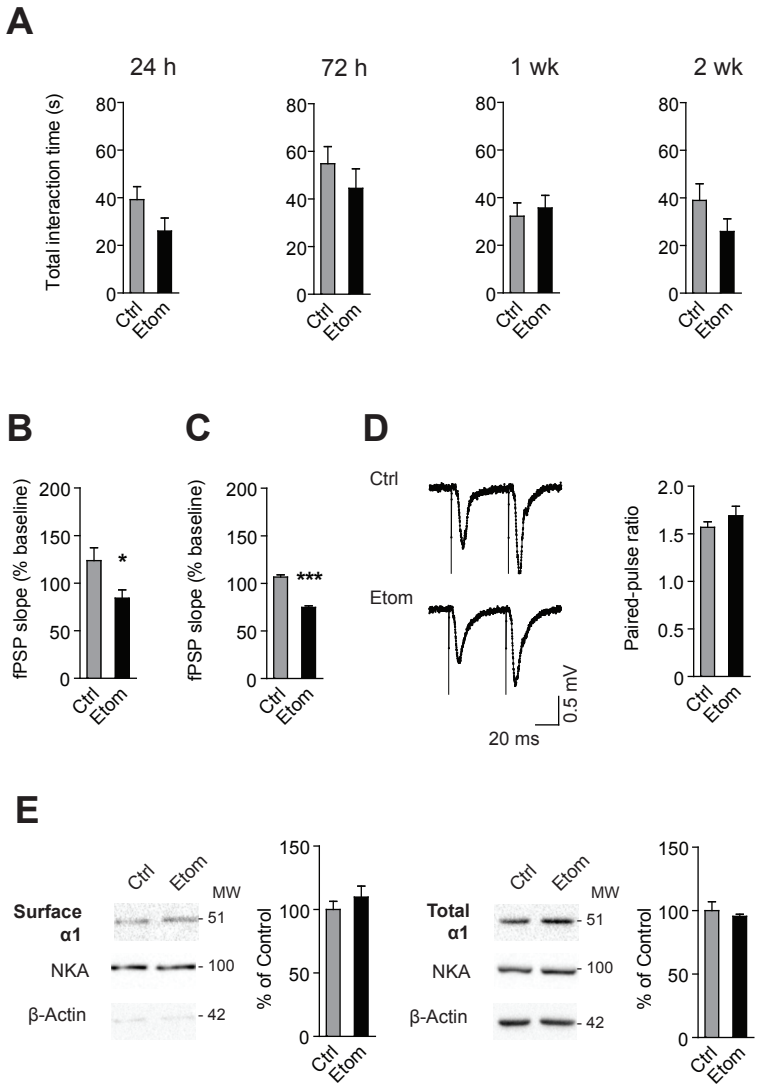
The effects of etomidate (8 mg/kg, i.p.) on post-tetanic depression, short-term depression and paired-pulse facilitation of fPSPs. (**A**-**C**) 24 h after etomidate treatment (*n* = 7), (**A**) post-tetanic depression and (**B**) short-term depression are observed in slices from etomidate-treated mice but (**C**) paired-pulse facilitation is not affected by etomidate treatment. (**D**- **F**) 1 week after etomidate treatment (*n* = 9-10), (**D**) post-tetanic depression, (**E**) short-term depression, and (**F**) paired-pulse facilitation are similar in slices from vehicle and etomidate-treated mice. fPSP: field postsynaptic potential. An unpaired, two-tailed Student's *t*-test was used to compare between groups. Data are shown as mean ± SEM. * $P < 0.05$, *** $P < 0.001$



Supplementary Figure 2
Etomidate increases cell-surface expression of β3 subunits but does not change cell-surface expression of δ and α1 subunits 24 h after treatment. Western blots of cell-surface and total expression of (**A**) β3 subunits (n = 3), (**B**) δ subunits (Surface n = 6, Total n = 3), and (**C**) α1 subunits (n = 3). NKA, Na^+/K^+ ATPase. MW, molecular weight (kDa). Samples collected at different time points were run on separate gels. An unpaired, two-tailed Student's t-test was used to compare between groups at each time point. Data are shown as mean ± SEM. *P < 0.05

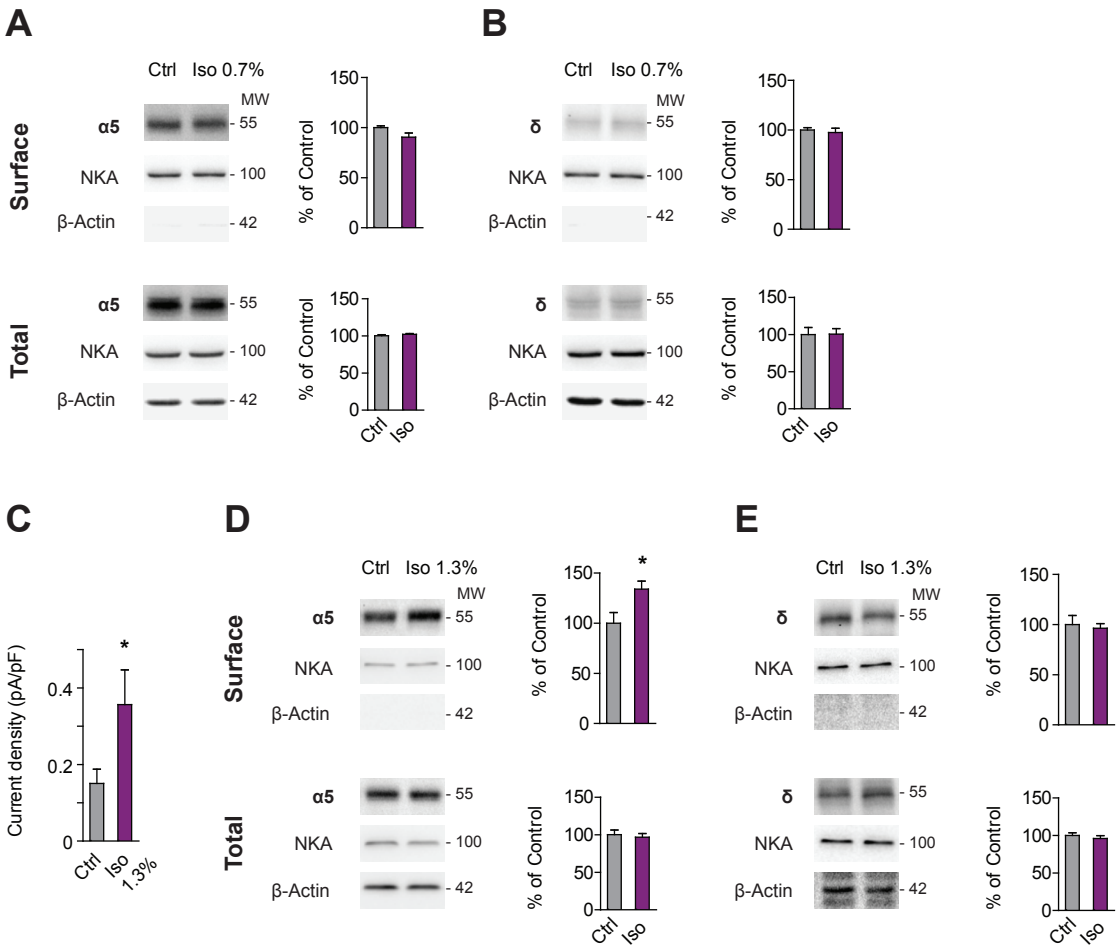


Supplementary Figure 3
Etomidate (8 mg/kg, i.p.) does not change total interaction time and does not affect plasticity of fPSPs in *Gabra5*$^{-/-}$ mice. (**A** and **B**) Total interaction time of (**A**) WT mice treated with L-655,708 24 h after etomidate treatment (*n* = 6-11, one-way ANOVA at each time point and Dunnett's post-test) and (**B**) *Gabra5*$^{-/-}$ mice (*n* = 8-13, one-way ANOVA at each time point and Dunnett's post-test). (**C**-**E**) 24 h after etomidate treatment of *Gabra5*$^{-/-}$ mice, (**C**) post-tetanic depression and (**D**) short-term depression are not observed and (**E**) paired-pulse facilitation is similar between groups (*n* = 7-8, for all analyses of fPSPs an unpaired, two-tailed Student's *t*-test was used). Ctrl: vehicle control, Etom: etomidate, Dex: dexmedetomidine. fPSP: field post-synaptic potential. Data are shown as mean ± SEM.



Supplementary Figure 4

The effect of an anesthetizing dose of etomidate (20 mg/kg i.p.) on total interaction time, plasticity, and expression of α1 subunits in WT mice. (**A**) Total interaction time with both objects during testing (*n* = 9-10). (**B**-**D**) 24 h after treatment (**B**) post-tetanic depression and (**C**) short-term depression are observed in slices from etomidate-treated mice but (**D**) paired-pulse faciliation is not affected by etomidate treatment (*n* = 6-7). (**E**) Western blots of cell-surface and total expression of α1 subunits (*n* = 4 mice). NKA, Na^+/K^+ ATPase. MW, molecular weight (kDa). An unpaired, two-tailed Student's *t*-test was used to compare between groups. Data are shown as mean ± SEM. * $P < 0.05$, *** $P < 0.001$.



Supplementary Figure 5

An anesthetizing dose of isoflurane increases the tonic current and cell-surface expression of α5GABA$_A$Rs 24 h after treatment. (**A** and **B**) Western blots of cell-surface and total expression of (**A**) α5 subunits and (**B**) δ subunits 24 h after a sedative dose of isoflurane (0.7%, 20 min; *n* = 3 mice).(**C**) Tonic current in WT slices (*n* = 9) 24 h after an anesthetizing dose of isoflurane (1.3%, 1 h; Mann-Whitney U test). (**D** and **E**) Western blot of cell-surface and total expression of (**D**) α5 subunits (*n* = 6) and (**E**) δ subunits (*n* = 5) 24 h after an anesthetizing dose of isoflurane (1.3%, 1 h). NKA, Na^+/K^+ ATPase. MW, molecular weight in kDa. An unpaired, two-tailed Student's *t*-test was used to compare between groups. Data are shown as mean ± SEM. **P* < 0.05

	Amplitude (pA)	Frequency (Hz)	Rise-time (ms)	Decay-time (ms)	Area (pA·ms)
Ctrl	35.49 ± 1.46	3.24 ± 0.53	4.01 ± 0.24	10.92 ± 0.75	372.56 ± 16.92
Etom	38.52 ± 1.19	3.24 ± 0.29	3.67 ± 0.22	9.96 ± 0.63	390.92 ± 19.33

Supplementary Table 1. Parameters of GABAergic miniature inhibitory postsynaptic currents 24 h after etomidate. Data are shown as mean ± SEM. n = 10-11, $P > 0.05$, An unpaired, two-tailed Student's t-test was used to compare between Ctrl and Etom groups.