Original Article

# Neonatal Propofol and Etomidate Exposure Enhance Inhibitory Synaptic Transmission in Hippocampal Cornus Ammonis 1 Pyramidal Neurons

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## Abstract

**Background:** Propofol and etomidate are the most important intravenous general anesthetics in the current clinical use and that mediate gamma-aminobutyric acid's (GABAergic) synaptic transmission. However, their long-term effects on GABAergic synaptic transmission induced by neonatal propofol or etomidate exposure remain unclear. We investigated the long-term GABAergic neurotransmission alterations, following neonatal propofol and etomidate administration.

**Methods:** Sprague-Dawley rat pups at postnatal days 4–6 were underwent 6-h-long propofol-induced or 5-h-long etomidate-induced anesthesia. We performed whole-cell patch-clamp recording from pyramidal cells in the cornus ammonis 1 area of acute hippocampal slices of postnatal 80–90 days. Spontaneous and miniature inhibitory GABAergic currents (spontaneous inhibitory postsynaptic currents [sIPSCs] and miniature inhibitory postsynaptic currents [mIPSCs]) and their kinetic characters were measured. The glutamatergic tonic effect on inhibitory transmission and the effect of bumetanide on neonatal propofol exposure were also examined.

**Results:** Neonatal propofol exposure significantly increased the frequency of mIPSCs (from 1.87 ± 0.35 Hz to 3.43 ± 0.51 Hz, $P < 0.05$) and did not affect the amplitude of mIPSCs and sIPSCs. Both propofol and etomidate slowed the decay time of mIPSCs kinetics (168.39 ± 27.91 ms and 267.02 ± 100.08 ms vs. 68.18 ± 12.43 ms; $P < 0.05$). Bumetanide significantly blocked the frequency increase and reversed the kinetic alteration of mIPSCs induced by neonatal propofol exposure (3.01 ± 0.45 Hz and 94.30 ± 32.56 ms).

**Conclusions:** Neonatal propofol and etomidate exposure has long-term effects on inhibitory GABAergic transmission. Propofol might act at pre- and post-synaptic GABA receptor A ($GABA_A$) receptors within GABAergic synapses and impairs the glutamatergic tonic input to GABAergic synapses; etomidate might act at the postsynaptic site.

**Key words:** Etomidate; Gamma-aminobutyric Acid; Glutamate; Propofol; Synapse Transmission

## Introduction

Propofol and etomidate are the most common intravenous general anesthetics in the current clinical use, and both can induce long-term behavioral effects, especially with early or elderly exposure.[1,2] Recently, a body of studies has been focused on the impact of anesthetics and surgery on neurodevelopment.[3,4] Briner *et al*.[5] found that neonatal propofol exposure decreased dendritic spine density and this modification persisted up to postnatal 90 days. Propofol impaired the adult neurogenesis in the hippocampus of mice in a developmental stage-dependent manner in mice.[6] Neonatal propofol exposure induced cognitive impairment persisting into adulthood in mice and rats, but the exact mechanism of long-term neurotoxicity is still unclear.[7,8]

Our previous study showed that neonatal exposure to sevoflurane induced acute seizure-like activity and was blocked by bumetanide (a $Na^+$-$K^+$-$2Cl^-$ cotransporter

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.193459

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**Received:** 09-06-2016 **Edited by:** Peng Lyu
**How to cite this article:** Zhang JQ, Xu WY, Xu CQ. Neonatal Propofol and Etomidate Exposure Enhance Inhibitory Synaptic Transmission in Hippocampal Cornus Ammonis 1 Pyramidal Neurons. Chin Med J 2016;129:2714-24.

1 [NKCC1] inhibitor) through reversing sevoflurane-induced apoptosis.[9] Our recent studies found that neonatal propofol or etomidate exposure caused seizure-like electroencephalogram activity and long-term (postnatal 70–80 days) impairment of prepulse inhibition (a type of startle test). However, its cellular mechanism needs to be further explored.

Glutamate and gamma-aminobutyric acid (GABA) are the two most abundant neurotransmitters in the brain, and their balanced activation of excitatory and inhibitory functions maintains the equilibrium of neuronal networks. Synaptic transmission by endogenous neurotransmitters via autoreceptors or heteroreceptors is modulated within neuronal circuits. Most studies have been done on the acute effect of propofol or etomidate on gamma-aminobutyric acid's (GABAergic) and glutamatergic neurotransmission in acute isolated neurons or brain slices.[10,11] However, the chronic actions of propofol and etomidate on GABAergic and glutamatergic synapses were less studied. Neuronal GABA receptor A ($GABA_A$) receptors are pentameric chloride ion channels, with synaptic $\alpha\beta\gamma$ and extrasynaptic $\alpha\beta\delta$ isoforms mediating phasic and tonic inhibitions, respectively, whose different subunits arrangement determines individual functions.[12] Phasic inhibition is further subdivided into fast $GABA_A$ ($GABA_{A,fast}$) and slow $GABA_A$ ($GABA_{A,slow}$) inhibitory postsynaptic currents (IPSCs). Propofol not only acts at pre- and post-synaptic $GABA_A$ receptors within GABAergic synapses but also increases extrasynaptic GABA responses.[11] Etomidate modulates $GABA_{A,slow}$ more strongly than $GABA_{A,fast}$ IPSCs, contributing to etomidate-induced, hippocampus-dependent memory impairment.[13] Our previous study showed that non-N-methyl-D-aspartate (non-NMDA) receptors (glutamate receptor 5 [GluR5]-containing kainate receptor) regulate the inhibitory synaptic transmission through endogenous glutamate, which is involved in age-dependent cognitive decline.[14] However, the research on long-term effects of neonatal propofol and/or etomidate on inhibitory and excitatory neurotransmissions interplay is very sparse, especially concerning the effects of propofol and/or etomidate on the interplay between GABAergic and glutamatergic neurotransmissions. In this study, we explored the effects of neonatal propofol and etomidate exposure on GABA-mediated synaptic inhibition and glutamate receptors' inputs on GABAergic synapses in hippocampal pyramidal neurons in the postnatal day 80–90 rats using electrophysiological recordings of acute hippocampal slices. These experiments were designed to address the following questions: (1) Does neonatal propofol or etomidate exposure have long-term effects on GABAergic neurotransmission? (2) Does neonatal exposure to propofol or etomidate affect the endogenous glutamatergic drive to inhibitory synaptic transmission? (3) Do propofol and etomidate share similar action mechanisms? (4) Does bumetanide have an effect on inhibitory transmission induced by neonatal propofol exposure?

# Methods

## Animals

All experimental procedures were approved by the University of Florida, the Institutional Animal Care and Use Committee (Gainesville, FL, USA). Sprague-Dawley rats were studied, and animals were housed under controlled illumination (12-h light/dark, lights on at 7:00 a.m.) and temperature (23°C–24°C) with free access to food and water (Protocol ID. 20134424). Within 24 h of delivery, litters were culled to 12 pups. At the age of 21 days, pups were weaned and housed in sex-matched groups of two for the rest of the study. To control for litter variability, we used several pups from each litter for different treatment conditions. Multiple sets of animals were used in a majority of the experiments.

## Drugs

Propofol and etomidate were purchased from APP Pharmaceuticals, LLC (Schaumburg, IL, USA), and Hospira, Inc. (Lake Forest, IL, USA), respectively. Tetrodotoxin (TTX) and lidocaine N-ethyl bromide (QX314) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Bumetanide (Ben Venue Laboratories, Inc.) was purchased from Bedford Laboratories (Bedford, OH, USA). DL-2-amino-5-phosphonovaleric acid (AP5) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were purchased from Tocris Cookson Inc. (Ellisville, MO, USA).

## Treatment groups

Postnatal day 4, 5, or 6 (P4–P6) rat pups of both genders were kept in a thermostated chamber (+37°C) with a continuous supply of oxygen (1.5 L/min) during anesthesia and/or maternal separation. Anesthesia protocol with propofol at 40 mg/kg intraperitoneally for induction for the first 60 min and then 20 $mg \cdot kg^{-1} \cdot h^{-1}$ intraperitoneally for maintenance for 5 h; this is a shorter version of what was originally described by Briner *et al*.[5] Neither Briner *et al*.[5] nor other authors[15] using a single injection of propofol at 75 mg/kg intraperitoneally detected significant changes in blood gases or glucose in neonatal rats. The etomidate group received 8 mg/kg etomidate intraperitoneally for induction, followed by 4 mg/kg intraperitoneally every hour for maintenance for 5 h. These doses of etomidate, which were assumed to be approximately equipotent to those of propofol, were chosen based on a study in adult rats comparing propofol and etomidate requirements during continuous infusion for achieving comparable levels of burst suppression.[16] The bumetanide group received bumetanide (1.82 mg/kg, intraperitoneally) 15 min prior to initiation of anesthesia with propofol as described above. Control animals received equal numbers and volumes of intraperitoneal injections of saline. All rat pups were separated from the dams for 5 h, except for rats in the negative control groups, which were not subject to maternal separation or any injections.

## Hippocampal slices preparation and electrophysiological recordings

The brain hippocampal slices were prepared from >P80 rats of all groups.[14] Briefly, the rat was decapitated and its brain

was removed after sedation with isoflurane; transverse hippocampal slices (250–300 micrometer, thick) were cut in ice-cold sucrose-artificial cerebrospinal fluid (aCSF) with a VT1000S microtome (Leica, Deerfield, IL, USA). One to four slices were recorded from one rat and one cell was recorded in one slice. At least one slice was used in every rat in every group. Slices were transferred immediately into a holding chamber and incubated at 32°C–33°C for a 30 min recovery period in aCSF containing the following (in mmol/L): 128 NaCl, 10 D-glucose, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgSO_4$, 3 KCl, and 1.25 $NaH_2PO_4$. Slices were then placed on a nylon mesh, submerged in normal aCSF bubbled with 95% $O_2$/5% $CO_2$ continuously and maintained at room temperature (about 21°C–24°C) until whole-cell patch-clamp recording (30 min to 5 h).

Cornus ammonis 1 (CA 1) pyramidal cells and interneurons were identified visually using an Axioskop 2FS microscope (Thornwood, NY, USA) equipped with a ×40 water-immersion objective coupled with an infrared differential interference contrast camera system. Whole-cell patch-clamp recordings were established using an Axopatch 200B (Axon Instruments, Union City, CA, USA). Membrane current and potential signals were digitized and analyzed with Digidata 1322A and pClamp 10.3 systems (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes of ≈5 MΩ were pulled with a P-1000 Sutter puller (Novato, CA, USA). The pipette solution had the following composition (in mmol/L) unless otherwise stated: 140 KCl, 0.1 $CaCl_2$, 5 EGTA, 10 HEPES, 4 ATP-$Mg^{2+}$, 0.4 GTP-2$Na^+$, and 1 QX314, pH 7.2, and 290 mOsm. QX314 was added to the pipette solution to block the $GABA_B$-mediated currents and to prevent the generation of a $Na^+$-dependent action potential. Spontaneous excitatory postsynaptic currents (sEPSCs) were excluded from recordings by adding glutamate receptor antagonists DNQX (20 μmol/L) and AP5 (20 μmol/L); therefore, all of the recorded inward currents were spontaneous IPSCs (sIPSCs).

### Acquisition and analysis of synaptic currents

Spontaneously occurring synaptic currents were filtered at 2 kHz and were digitized at 10 kHz using Digidata 1322A. Synaptic currents were collected at 5 min for each experimental condition. Offline analysis of synaptic currents was performed using the Mini-Analysis software (Version 6.0; Synaptosoft, Decatur, GA, USA). Synaptic currents were screened automatically using an amplitude threshold of 10 pA. Events were then visually screened to ensure that the analysis was not distorted by changes in noise level or by membrane fluctuations. If the background noise increased during the recording, the data from that cell were discarded. The data generated from these measurements were used to plot cumulative probability amplitude and interevent interval graphs, with each distribution normalized to a maximal value of one. Cumulative probability plots obtained under different experimental conditions were compared using the nonparametric Kolmogorov-Smirnov (K-S test), which estimates the probability that two cumulative distributions differ from each other by chance alone.[14]

### Method of kinetic changes of miniature inhibitory postsynaptic currents

Offline analysis of miniature inhibitory postsynaptic currents (mIPSCs) kinetic parameters was performed using Mini-Analysis 6.0 (Synaptosoft Inc., Decatur, GA, USA). Generally, individual currents >10 pA could be clearly distinguished above baseline noise in the 5-min current traces collected from individual neurons. For mIPSCs decay analysis, low noise traces and nonoverlapping events were used to generate an ensemble averaged mIPSCs by aligning currents on the rising phase. The 10–90% decay phase of this average for each neuron was fitted with a biexponential function:

$$Y(t) = A1 \exp(-t/\tau 1) + A2 \exp(-t/\tau 2) + As.$$

where A1 and A2 are the fraction of the fast and slow decay components, respectively, As is the steady-state current, and τ1 and τ2 are the fast and slow decay time constants, respectively. The slow time constant of decay of $GABA_{A,slow}$ (30–70 ms) indicates τ2 as opposed to 3–8 ms for $GABA_{A,fast}$ (τ1).[13] Miniature and/or spontaneous inhibitory synaptic current events of each neuron were analyzed and averaged; then, the kinetic indices of different neurons in the same groups were averaged, respectively.

### Statistical analysis

Values are reported as mean ± standard error of the mean (SEM). SigmaPlot 12.5 software (Systat Software, Inc., Point Richmond, CA, USA) was used for statistical analyses. Single comparisons were tested using the Student's *t*-test, whereas multiple comparisons among groups were analyzed using analysis of variance, followed by Holm-Sidak test. All comparisons were run as two-tailed tests. A value of $P < 0.05$ was considered statistically significant.

## Results

### Neonatal propofol exposure but not etomidate increases the frequency of miniature inhibitory postsynaptic currents but not amplitude in p80–90 rats

To understand the long-term effect of neonatal propofol and etomidate on GABAergic neurotransmission, we performed patch-clamp recordings of CA1 pyramidal neurons in the presence of DNQX (20 μmol/L) and AP5 (20 μmol/L, NMDA receptor antagonist). sIPSCs and mIPSCs changes after the neonatal exposure were recorded and confirmed by the application of the $GABA_A$ receptor antagonist bicuculline. There was no significant difference in the mean frequency or amplitude of sIPSCs among the control, propofol, or etomidate groups. The mean sIPSCs frequency and amplitude of control group rats were 5.12 ± 0.79 Hz and 47.95 ± 4.75 pA ($n$ = 8); the mean sIPSCs frequency and amplitude in the propofol group

were 5.52 ± 0.72 Hz and 42.97 ± 2.9 pA ($n$ = 9); the mean sIPSCs frequency and amplitude in the etomidate group were 3.87 ± 0.62 Hz and 38.63 ± 3.43 pA ($n$ = 9). However, the mean frequency of mIPSCs, but not amplitude, was significantly higher in the propofol exposure group compared with the control or etomidate groups ($P < 0.05$, unpaired Student's $t$-test, Figure 1e and 1f). The representative traces of mIPSCs are shown in Figure 1a. The cumulative frequency, but not amplitude histograms, for representative propofol exposure and control cells [Figure 1b] demonstrated a significant increase in the frequency but not amplitude of mIPSCs with propofol exposure (K-S test, $P < 0.0001$). The cumulative frequency and amplitude histograms for representative etomidate exposure and control cells [Figure 1c] demonstrated no significant difference between the etomidate and control groups (K-S test, $P > 0.05$). As shown in Figure 1e and 1f, the mean mIPSCs frequency and amplitude in control group rats were 1.87 ± 0.35 Hz and 27.63 ± 2.84 pA ($n$ = 8), the frequency and amplitude in the propofol group were 3.43 ± 0.51 Hz and 28.82 ± 1.89 pA ($n$ = 9), and the frequency and amplitude of etomidate group were 1.89 ± 0.41 Hz and 28.5 ± 2.12 pA ($n$ = 9).

## Neonatal propofol and etomidate exposure slows the decay of slow gamma-aminobutyric acid receptor a miniature inhibitory postsynaptic currents in p80–90 rats

To test the kinetic changes of mIPSCs after neonatal propofol and etomidate exposure, 10–90% rise time, area, half width, $\tau_{fast}$, and $\tau_{slow}$ were measured [Figure 2]. Propofol and etomidate neonatal exposure significantly slowed the time constant of decay of $\tau_{slow}$ but not $\tau_{fast}$ ($P < 0.05$). The time constant of $\tau_{slow}$ in the control, propofol, and etomidate groups was 68.18 ± 12.43 ms, 168.39 ± 27.91 ms, and 267.02 ± 100.08 ms, respectively [Figure 2e]. The time constant of $\tau_{fast}$ in control, propofol, and etomidate groups was 7.68 ± 0.86 ms, 7.02 ± 1.32 ms, and 9.51 ± 1.16 ms, respectively [Figure 2d]. There was no significant difference among the three groups in 10–90% rise time and half width of mIPSCs. Ten percent to 90% rise time in the control, propofol, and etomidate groups was 1.00 ± 0.13 ms, 0.96 ± 0.25 ms, and 1.27 ± 0.27 ms, respectively [Figure 2a]. Half-width of the control, propofol, and etomidate groups was 6.91 ± 0.67 ms, 7.02 ± 1.32 ms, and 9.51 ± 1.20 ms, respectively [Figure 2c]. There was a significant increase in area of mIPSCs in the propofol group compared with control and etomidate groups. Area indicates charge (charge [Q] = current × time, in pico-Coulomb [pC]) and the approximate average number of GABAergic vesicle release. The area of the control, propofol, and etomidate groups was 311.72 ± 21.06 pC, 572.40 ± 130.34 pC, and 422.09 ± 81.62 pC, respectively [Figure 2b]. These data suggest that propofol and etomidate induce changes in mIPSCs kinetics through the postsynaptic pathway. Propofol-induced area increase might be involved in the presynaptic pathway in GABAergic modification. The kinetics of sIPSCs were measured and there were no significant differences among the three groups (data not shown).

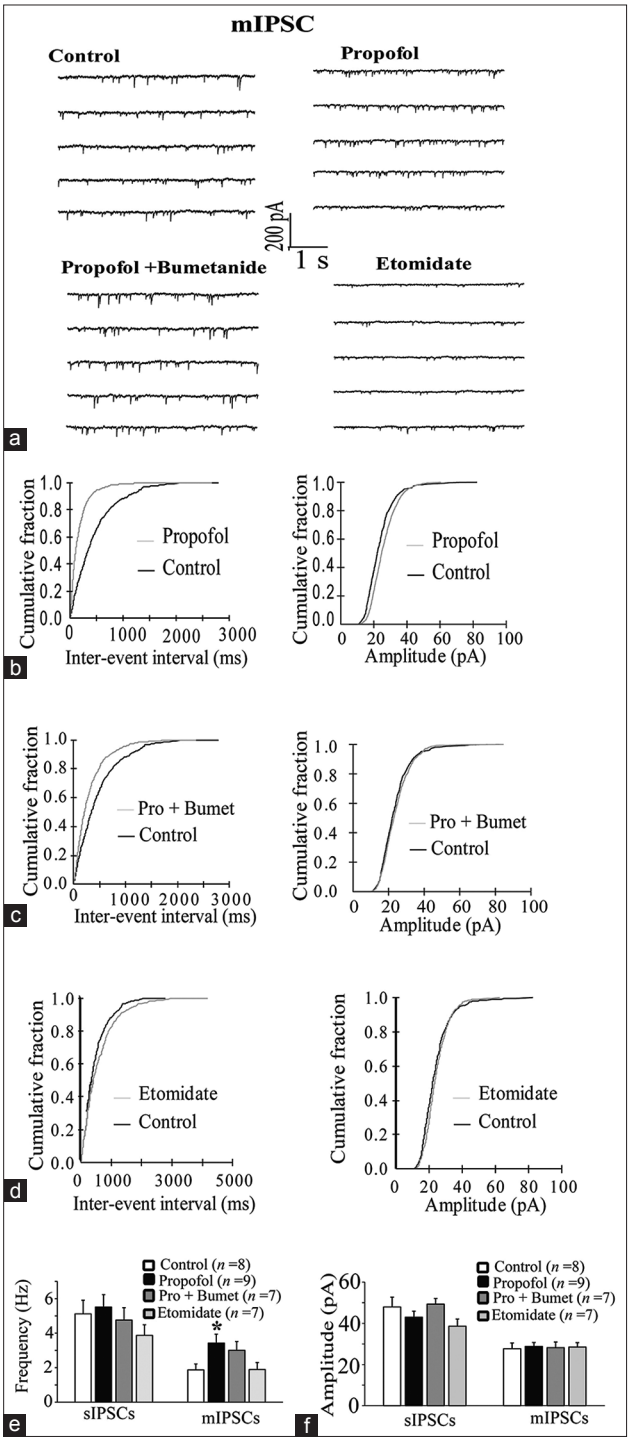


**Figure 1:** Frequency but not amplitude of mIPSCs in CA1 neurons increased after neonatal propofol exposure and blocked by bumetanide in postnatal 80–90 days' rats. (a) Representative traces of mIPSCs. (b) Cumulative frequency and amplitude distributions of mIPSCs based on data shown in (a, $P < 0.0001$). (c and d) No statistically different between the two groups. (e and f) Bar graphs show that the mean frequency of sIPSCs and mIPSCs ($P < 0.05$, Student's $t$-test compared with control group). mIPSCs: Miniature inhibitory postsynaptic currents; sIPSCs: Spontaneous inhibitory postsynaptic currents, CA1: Cornus ammonis 1; Pro: Propofol; Bumet: Bumetanide.

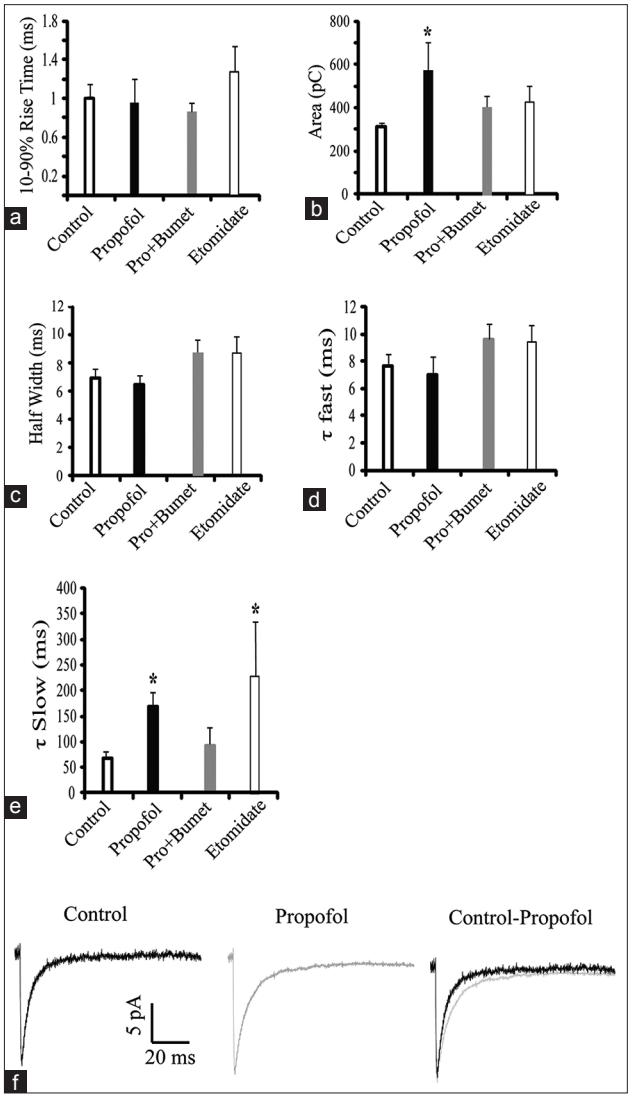


**Figure 2:** Neonatal propofol and etomidate slow the decay of $GABA_{A,slow}$ mIPSCs in postnatal 80–90 rats. (a-e) Bar charts summarize the mean values of the rise time, area, half width, $\tau_{fast}$, and $\tau_{slow}$ in control ($n = 9$), propofol ($n = 8$), propofol + bumetanide ($n = 5$), and etomidate ($n = 9$) groups, respectively, showing propofol and etomidate significantly slow the $\tau_{slow}$ and propofol also significantly increases the charge area (Student's *t*-test, $*P < 0.05$). (f) Representative decay kinetics of mIPSCs in control and after propofol exposure. mIPSCs: Miniature inhibitory postsynaptic currents; Pro: Propofol; Bumet: Bumetanide; pC: pico-Coloumb.

## Neonatal propofol exposure impairs glutamatergic tonic regulation of spontaneous inhibitory synaptic transmission

As the above results suggest, propofol medicates GABAergic neurotransmission through pre- and post-synaptic pathways and etomidate regulates GABAergic neurotransmission through a postsynaptic mechanism. Our previous study showed that GluR5-containing kainate receptors (non-NMDA glutamate receptor) regulate the inhibitory synaptic transmission through endogenous glutamate; therefore, we tested how neonatal propofol and etomidate exposure affect endogenous glutamatergic tonic regulation to inhibitory synaptic transmission in P90 approximately rats.[17] To determine whether NMDA receptors are involved in the tonic glutamatergic input on GABAergic neurotransmission, we tested the effect of AP5, a selective NMDA receptor antagonist, on sIPSCs. Seven neurons from the control group, six neurons from neonatal propofol exposure group, and seven neurons from the neonatal etomidate exposure group were tested with AP5 (20 μmol/L) and then with AP5 plus DNQX (10 μmol/L, a selective non-NMDA receptor antagonist). There were no significant changes in the frequency and amplitude of sIPSCs, following the bath application of AP5 ($P > 0.05$, student's paired *t*-test). However, DNQX plus AP5 induced increases in sIPSC frequency in the control [from 5.81 ± 1.09 Hz to 10.8 ± 2.25 Hz, $P < 0.05$, $n = 7$; Figure 3a and 3c] and etomidate group [from 4.92 ± 0.92 Hz to 7.66 ± 1.52 Hz, $P < 0.05$, $n = 7$; Figure 3c] but not in the propofol group [from 5.19 ± 0.93 Hz to 6.32 ± 1.17 Hz, $P > 0.05$, $n = 6$; Figure 3b and 3c]. There were no significant changes in amplitude of sIPSCs, following the bath application of AP5 plus DNQX in the control, propofol, and etomidate groups [63.29 ± 6.79 pA, 60.72 ± 12.2 pA, and 56.48 ± 13.5 pA, respectively, Figure 3c]. To further evaluate the differences among these three groups, we calculated the ratio of frequency, or amplitude, following DNQX application versus that before DNQX application [Figure 4]. The ratio of frequency and amplitude was significantly lower in the propofol group than the control group [Student's *t*-test, $P < 0.05$; Figure 4a–4c]. The mean value of the frequency and amplitude ratio in the propofol group was 1.25 ± 0.10 and 0.89 ± 0.03 ($n = 6$), respectively. The mean value of the frequency and amplitude ratio in the control groups was 1.83 ± 0.13 and 1.13 ± 0.09 ($n = 7$), respectively. There was no significant difference between the control and etomidate groups in frequency ratio and amplitude ratio. The mean value of the frequency and amplitude ratio in the etomidate group was 1.58 ± 0.19 and 0.94 ± 0.08, respectively [Figure 4a–4c]. These results demonstrate that neonatal propofol exposure impaired the regulation of inhibitory synaptic input by endogenous glutamate through the non-NMDA receptor.

## Neonatal propofol exposure regulates miniature inhibitory postsynaptic currents via an action potential-dependent and/or potential-independent presynaptic mechanism

To determine whether non-NMDA receptors regulate sIPSCs or mIPSCs presynaptically or postsynaptically, we examined the effects of DNQX on mIPSCs. Thirty-seven hippocampal CA1 neurons were tested (12 for control, 10 for propofol, and 15 for etomidate) in the presence of TTX (1 μmol/L) and AP5 (20 μmol/L). As shown in Figure 5, DNQX had no effect on mIPSCs' frequency and amplitude in the control and etomidate groups. The frequency and amplitude of mIPSCs before DNQX were 2.63 ± 0.39 Hz and 35.27 ± 1.78 pA, respectively, and

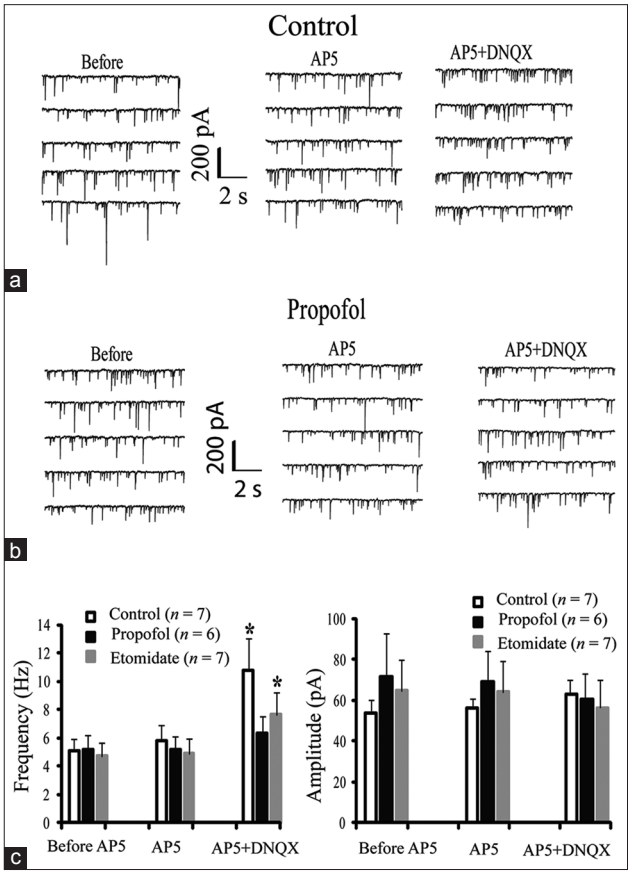


**Figure 3:** Non-NMDA receptor-mediated glutamatergic tonic regulation of sIPSCs in CA1 pyramidal neurons was impaired by neonatal propofol exposure but not etomidate. (a) DNQX but not AP5 increased frequency but not amplitude of sIPSCs. (b) AP5 and DNQX had no effects on sIPSCs following neonatal propofol exposure. (c) Bar graph shows that DNQX significantly increased the frequency of sIPSCs in control and etomidate (*$P < 0.05$). NMDA: N-methyl-D-aspartate; sIPSCs: Spontaneous inhibitory postsynaptic currents; CA1: Cornus ammonis 1; DNQX: 6,7-dinitroquinoxaline-2,3-dione; AP5: DL-2-amino-5-phosphonovaleric acid.

the frequency and amplitude of mIPSCs after DNQX application were 2.19 ± 0.36 Hz and 33.58 ± 2.04 pA, respectively ($n$ = 15). However, there was a significant difference in the propofol group between before and after DNQX application (Student's paired $t$-test, $P < 0.05$; $n = 10$). The mean frequency and amplitude of mIPSCs before DNQX application were 5.22 ± 0.74 Hz and 38.07 ± 1.97 pA, respectively, and the frequency and amplitude of mIPSCs after DNQX application were 3.54 ± 0.47 Hz and 29.83 ± 1.96 pA, respectively [Figure 5b and 5c]. The DNQX significantly reduced the frequency and amplitude of mIPSCs further confirmed that non-NMDA receptor-mediated inhibition transmission was impaired. This result is somehow unexpected, but it suggests that there is a non-NMDA receptor-mediated glutamatergic tonic excitation on GABAergic neurotransmission in neonatal propofol exposure. Therefore, we can conclude that neonatal propofol exposure might turn glutamatergic tonic inhibition into excitation direction.

### Bumetanide blocks the frequency increase and kinetic alteration of miniature inhibitory postsynaptic currents induced by neonatal propofol exposure

Previous studies have shown that bumetanide (the NKCC1 inhibitor) can block neonatal propofol- or sevoflurane-induced seizure activity in rats, leading us to explore the effect of bumetanide on inhibitory transmission after neonatal propofol exposure. Pretreatment with bumetanide before propofol exposure had no effect on sIPSCs frequency and amplitude compared with the control or propofol groups ($P > 0.05$, unpaired Student's $t$-test). The frequency and amplitude of sIPSCs were 4.76 ± 0.81 Hz and 49.29 ± 2.77 pA, respectively [$n = 7$, Figure 1a, 1e, and 1f]. However, pretreatment with bumetanide significantly blocked the frequency increase of mIPSCs induced by neonatal propofol exposure, but not amplitude, compared with the propofol group ($P < 0.05$, unpaired Student's $t$-test). The frequency and amplitude of mIPSCs were 3.01 ± 0.45 Hz and 28.21 ± 2.72 pA, respectively [$n$ = 7, Figure 1a, 1e, and 1f]. The time constant of decay of $\tau_{slow}$ of bumetanide in combination with propofol group was 94.30 ± 32.56 ms ($n$ = 7). Bumetanide significantly reduced the time constant of decay of $\tau_{slow}$ compared with the propofol group [$P < 0.05$, unpaired Student's $t$-test, Figure 2e]. There were no significant differences among the control, propofol, and bumetanide combined with the propofol group in 10–90% rise time, half width, area, and decay $\tau_{fast}$ of mIPSCs. The 10–90% rise time, half width, area, and $\tau_{fast}$ of mIPSCs were 0.86 ± 0.10 ms, 8.75 ± 0.89 ms, 404.53 ± 50.74 pC, and 9.64 ± 1.24 ms, respectively [Figure 2a]. The kinetics of sIPSCs were measured and found that there were no significant differences among the control, propofol, and bumetanide in combination with propofol group (data not shown). These results suggest that bumetanide blocked the frequency increase and kinetic alteration of mIPSCs induced by neonatal propofol exposure.

## Discussion

In this study, we used whole-cell patch-clamp recordings of acute hippocampal slices to examine long-term effects on the interaction of GABAergic and glutamatergic neurotransmission in postnatal 80–90 days rats, following neonatal propofol or etomidate exposure. We found that neonatal propofol, but not etomidate, exposure significantly increases the frequency of mIPSCs but not sIPSCs frequency and does not affect the amplitude of mIPSCs or sIPSCs. However, both propofol and etomidate slow the decay time of mIPSCs kinetics. DNQX has no effect on sIPSCs but significantly reduces the frequency and amplitude of mIPSCs, following propofol exposure. In addition, bumetanide significantly blocks the frequency increase and reverses the kinetic alteration of mIPSCs induced by neonatal propofol exposure. In the discussion below, we focus on: (1) Propofol increases IPSC through pre- and post-synaptic pathways. (2) Etomidate mediates IPSC

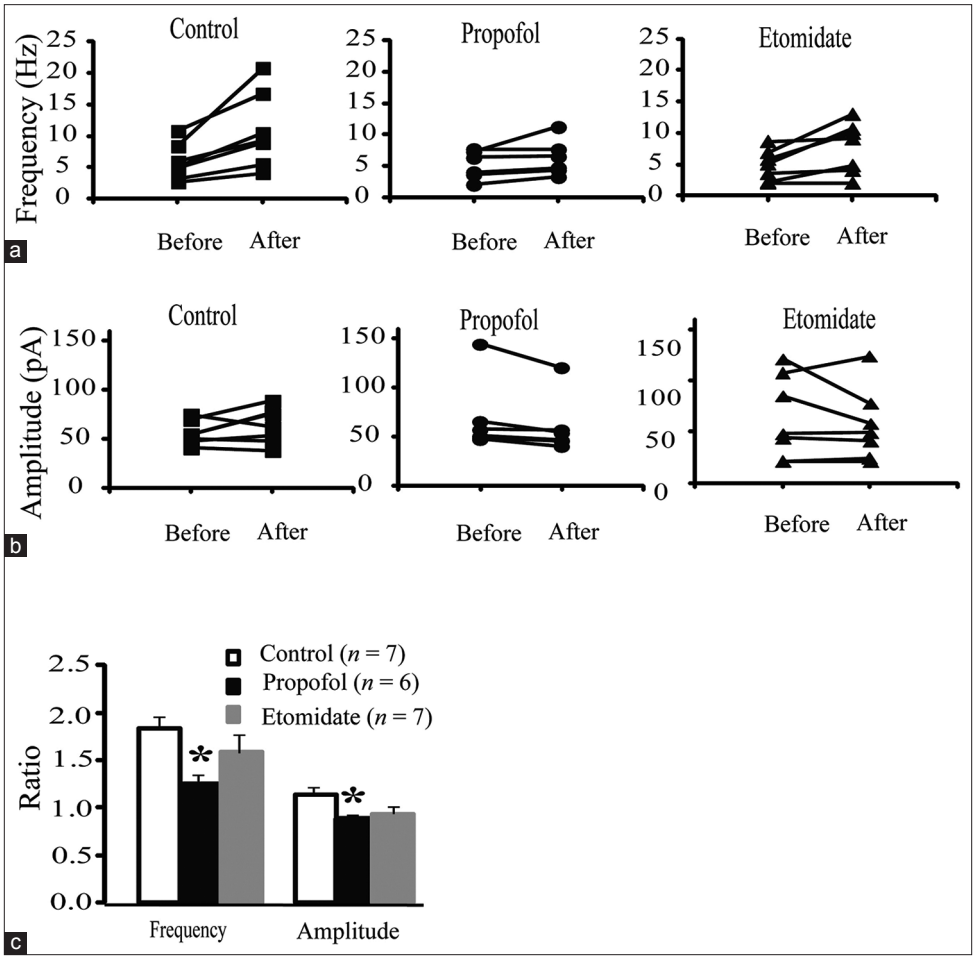


**Figure 4:** Effects of DNQX on sIPSCs in CA1 neurons in control, propofol, and etomidate exposure rats. (a and b) sIPSCs frequencies and amplitudes of individual cells before and after DNQX (10 μmol/L) application. (c) Bar chart shows that the ratio changes in frequency and amplitude of sIPSCs induced by DNQX in three groups. The changes of sIPSCs frequency and amplitude in neonatal propofol exposure are significantly different to that obtained in control group (Student's *t*-test; $*P < 0.05$). DNQX: 6,7-dinitroquinoxaline-2,3-dione; sIPSCs: Spontaneous inhibitory postsynaptic currents; CA1: Cornus ammonis 1.

through a postsynaptic pathway. (3) Effects of neonatal propofol and etomidate exposure on the interactions of GABAergic and glutamatergic neurotransmission. (4) Effect of bumetanide on GABAergic neurotransmission after neonatal propofol exposure.

## Propofol increases inhibitory postsynaptic current through pre- and post-synaptic pathways

In our study, neonatal propofol exposure induced long-term effect on GABAergic neurotransmission, and propofol increased the frequency of mIPSCs, slowed the decay time, and increased area of charge. Increased mIPSCs frequency indicates an alteration in the presynaptic terminal, leading to the increased probability of synaptic vesicle fusion and neurotransmitter release, whereas increased mIPSCs amplitude reflects an increase in postsynaptic receptor sensitivity to the released GABA, possibly due to changes in receptor subunit composition or number.[18] Propofol slows the decay time of mIPSCs, showing that propofol might act at postsynaptic sites. Kinetics such as time course of deactivation and desensitization of $GABA_A$ receptors are dependent on structural properties such as subunit units.

The mechanism of propofol on presynaptic GABA release includes: (1) Propofol induces $Ca^{2+}$-dependent GABA release. Propofol acts on an inward $Cl^-$ current in the GABAergic terminal, which initiates depolarization to trigger GABA release, which occurs through voltage-dependent $Ca^{2+}$ channels and induces a $Ca^{2+}$ increase in GABAergic terminals. Propofol might influence the $Ca^{2+}$/calmodulin protein kinase IIα pathway to trigger $GABA_A$ receptors and enhance inhibitory transmission by selective translocation.[19] (2) Propofol induces an action potential-independent GABA release. Our results show that neonatal propofol exposure has no effect on the frequency of sIPSCs but increases mIPSCs frequency. According to the quantum theory of vesicular release, mIPSCs are recorded in the presence of TTX and are assumed to represent the spontaneous release of individual vesicles or quanta of neurotransmitter from the presynaptic membrane. Therefore, we speculate that propofol might induce probability of synaptic vesicle fusion and neurotransmitter release.

The mechanism of propofol acting on postsynaptic $GABA_A$ receptor: neonatal propofol exposure slows the decay time

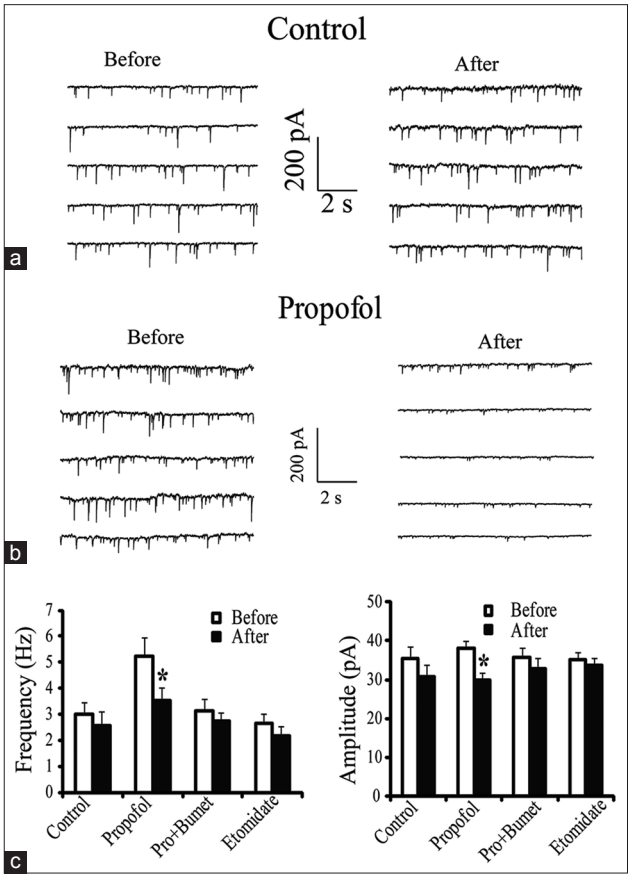


**Figure 5:** DNQX significantly decreased the frequency and amplitude of mIPSCs of CA1 pyramidal neurons in the presence of AP5 in propofol group but not in other groups. (a and b) Representative traces of mIPSCs obtained before (left) and after the application of 10 μmol/L DNQX (right) in control and propofol groups. (c) Bar graphs show the mean frequency or amplitude changes of mIPSCs in four groups (A paired Student's *t*-test, $*P < 0.05$). DNQX: 6,7-dinitroquinoxaline-2,3-dione; mIPSCs: Miniature inhibitory postsynaptic currents; CA1: Cornus ammonis 1; AP5: DL-2-amino-5-phosphonovaleric acid; Pro: Propofol; Bumet: Bumetanide.

and increases the charge transfer of kinetic characters of mIPSCs, which represents the postsynaptic effects. Our results show that propofol only affects the slow time constant of averaged mIPSCs, which is inconsistent with Drexler's report that propofol affects both the fast and slow time constants of averaged IPSCs.[10] They found that the slow time constant is dependent on β3 subunits and the fast time constant is related to α1 subunits, and they supported an idea that propofol and etomidate act via different $GABA_A$ receptor subtypes. It is interesting to note that the fast decay time constant was unaffected by neonatal propofol exposure. Normally, the fast decay is related to the rate of initial closing and rapid desensitization of the channel, whereas the slow decay is determined by inactivation of the channel after dissociation of the transmitter, recovery from desensitization, and the unbinding kinetics in the nondesensitization state. The possible mechanisms of propofol acting on postsynaptic $GABA_A$ receptors include: (1) Propofol affects the receptor subunit-dependent modification, which contributes to the development of changes of mIPSCs kinetics. $GABA_A$ receptor heterogeneity determines the kinetics of receptor deactivation and/or desensitization.[10] Propofol can act on β3-containing $GABA_A$ receptors and enhance $GABA_A$ receptor channel opening, prolong deactivation, and slow desensitization of $GABA_A$ receptor subunits. Propofol can also recruit new subunits such as α4 to the surface of a neuron via increasing α4 subunit mRNA.[20] (2) Neonatal propofol exposure might increase GABAergic synaptic cleft distance. A larger cleft distance would not only slow the kinetics but also decrease the mIPSCs amplitude. A developmental alteration in mIPSCs decay properties might be due to changes in synapse location. Propofol exposure in the early postnatal period rapidly induces changes in dendritic spine density and these effects are developmentally stage-dependent, persistent up to postnatal 90 days.[5] Propofol might induce the synapse move from soma to distal dendrites. (3) Propofol might mediate an inward $Cl^-$ current in terminal, which initiates depolarization to trigger GABA release.[21] (4) Propofol directly activates the $GABA_A$ receptor. At low concentrations, propofol enhances agonist-stimulated $GABA_A$ receptor activity, and high propofol concentration directly activates receptors.[22]

Several studies have reported that propofol increases extrasynaptic GABA responses; thus, propofol exposure might affect GABA tonic current.[11,23] Propofol mediates α5-containing $GABA_AR$, which is found at extrasynapses and co-assembled with β3-containing $GABA_AR$, which contributes to the "tail" kinetics of synaptic currents.[24,25] A previous study showed that the α5 $GABA_A$ receptor subunit can be assembled into synapses later during development and is responsible for the generation of a slow current.[26] However, GABA tonic alteration after neonatal propofol exposure was not explored in this study and might need further study.

## Etomidate mediates inhibitory postsynaptic currents through a postsynaptic pathway

Neonatal etomidate exposure did not affect the frequency or amplitude of sIPSCs and mIPSCs; however, it slowed the decay time of mIPSCs, which suggests that the long-term effect of etomidate on the $GABA_A$ receptor was via a postsynaptic pathway. There are several mechanisms of kinetic characters of mIPSCs induced by etomidate exposure: like propofol, etomidate might affect the receptor subunits co-assemble, subunits translocation, synaptic cleft distance enlargement or might directly activate $GABA_A$ receptors. The actions of etomidate also depend on $GABA_A$ receptor subunit makeup. Etomidate can activate the receptors containing β2 and/or β3 subunits. Its activation is also affected by other subunits such as the α or γ subunit.[27] There is a report that the sites where propofol binds to $GABA_A$ receptors differ from those of etomidate, and they might differentially engage different types of $GABA_A$ receptors.[28] Etomidate, the allosteric agonist, can also directly activate α1β2γ2L or α1β3γ2 $GABA_A$ receptors.[29,30] Previous reports have shown that acute application of clinical concentrations of etomidate in cultured postnatal hippocampal neurons or

*Xenopus* oocytes-expressed $GABA_A$ receptors ($\alpha1\beta2\gamma2L$) can slow the inhibitory postsynaptic current decay time constant mediated by $GABA_A$ receptors, prolonging postsynaptic inhibition, which occurs by enhancing gating and desensitization of $GABA_A$ receptors.[31] Interestingly, our study showed the long-term modification of etomidate exposure.

Like propofol, several studies reported that etomidate increases extrasynaptic GABA responses; thus, etomidate exposure might affect GABA tonic current.[31,32] Etomidate mediates $\alpha4$ subunit-containing and/or $\delta$-containing subunits, which are found at extrasynapses of $GABA_A$ receptors.[32-34] A previous study showed that etomidate targets the $\alpha5$ $GABA_A$ receptor subunit to regulate synaptic plasticity and memory blockade.[34] A recent report showed that etomidate more sensitively modulated a gender-specific enhancement of tonic inhibition current in the dentate gyrus, together with a deficit in long-term potentiation.[32] Therefore, one possible relationship is between neonatal etomidate exposure and the long-term effect of clinical cognitive impairment. Dai *et al*.[13] found that etomidate modulates slow IPSCs ($GABA_{A,slow}$) more strongly than fast IPSCs ($GABA_{A,fast}$) and prolongs the time constant of decay of both $GABA_{A,slow}$ and $GABA_{A,fast}$, which are involved in etomidate-induced amnesia. However, the GABA tonic alteration after neonatal etomidate exposure was not explored in this study and might need further study.

### Interaction of glutamatergic and gamma-aminobutyric acid's neurotransmission in neonatal propofol exposure

The study of network of interplay between glutamatergic and GABAergic neurotransmission has been recently emphasized. Our previous study showed that there is glutamatergic tonic inhibition on GABAergic neurotransmission through the non-NMDA receptor, GluR5-containing kainate receptor.[14] In the present study, DNQX, a non-NMDA glutamatergic antagonist, significantly induced the increase of sIPSCs in the control and etomidate exposure groups, which suggests that there is glutamatergic tonic inhibition on GABAergic neurotransmission; etomidate exposure did not affect the glutamatergic tonic inhibition on GABAergic neurotransmission. However, DNQX did not induce the increase of sIPSCs in the propofol group, which suggests that there is no glutamatergic tonic inhibition on GABAergic neurotransmission, or impaired this pathway. Interestingly, DNQX application significantly reduced the frequency and amplitude of mIPSCs in the neonatal propofol group, which suggested that propofol acted on pre- and post-synaptic sites via non-NMDA receptor-mediated glutamatergic tonic pathway. It is possible that the decreased mIPSCs frequency indicates a decreased probability of synaptic vesicle fusion and GABA release in the presynaptic terminals, whereas decreased mIPSCs amplitude reflects a decrease in postsynaptic receptor sensitivity to GABA and the change of $GABA_A$ receptor subunit composition or the number of receptors present.[35] Non-NMDA glutamate receptors include $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and kainate receptors. We cannot characterize which type of non-NMDA glutamate receptors is involved in the tonic drive received by sIPSCs or mIPSCs. Our previous study supported the idea that the kainate receptor is involved in this tonic drive. There is a report that kainate receptor-mediated transmission might be of significance in understanding the mechanism of propofol action at the excitatory and inhibitory amino acid receptors.[36] The possibility of AMPA receptor-mediated tonic is not precluded. The study about general anesthetics on ligand-gated ion channels showed that propofol significantly potentiates $GABA_A$ and glycine receptors and slightly inhibited AMPA and nicotinic acetylcholine receptors.[37] Propofol induced GABA and glutamate release in cerebrocortical synaptosomes, and the glutamate release is through presynaptic voltage-dependent $Na^+$ channels as a molecular target.[38] In our study, mIPSCs were recorded in the presence of 1 μmol/L TTX, which can block voltage-dependent $Na^+$ channels. Therefore, we conclude that neonatal propofol exposure alters the endogenous glutamate level in the synaptic cleft with TTX application. A recent report showed that GABAergic neurotransmission also modulated glutamatergic neurotransmission. The propofol-induced changes in excitatory postsynaptic currents (EPSCs) and evoked EPSCs resulted from presynaptic $GABA_A$ receptor-mediated depolarization.[11]

### Effect of bumetanide on gamma-aminobutyric acid's neurotransmission, following neonatal propofol exposure

Our previous study showed that bumetanide reversed sevoflurane-induced apoptosis and seizure activity.[9] Neonatal propofol exposure impaired neuronal synaptic density and induced long-term cognitive and behavioral abnormalities. In the present study, we found that neonatal propofol exposure enhanced long-term GABAergic neurotransmission. Normally, a precise balance of excitatory and inhibitory synaptic inputs is essential for the proper function of a neuronal circuit, and the increase of GABAergic neurotransmission is involved in the loss of learning and memory. Therefore, we deduced that long-term GABAergic neurotransmission changes induced by neonatal propofol exposure account for the cognitive impairment, following propofol exposure. It is interesting that bumetanide significantly blocked the frequency increase and the slowed the decay time constant of mIPSCs induced by neonatal propofol exposure. Bumetanide, a selective NKCC1 antagonist, is used to treat seizures but is still controversial.[17] The possible mechanisms of bumetanide acting on GABAergic neurotransmission include: (1) Propofol presynaptic actions depolarize GABAergic terminals to trigger GABA release, which might depend on the $Cl^-$ gradient. Bumetanide blocks the NKCC1-mediated an inward $Cl^-$ current in terminals, preventing the depolarization-induced GABA release.[21] (2) Bumetanide reverses the alteration of decay kinetics induced by propofol, which suggests that bumetanide alters postsynaptic $GABA_A$

receptors in the hippocampus. Bumetanide can restore dysfunctional $Cl^-$ homeostasis at postsynaptic sites in adult epileptic tissue.[39] There is some discrepancy with the report of Jin *et al*.[21] They found that furosemide, another NKCC cotransporter, reversibly blocked propofol-evoked IPSC frequency changes without altering waveforms, but our results show that bumetanide also reverses the alteration of decay time constant kinetic of mIPSCs. (3) Bumetanide blocks spontaneous network events. Giant depolarizing potentials (GDPs) are brain network activities that depend on GABAergic neurotransmission, thought to be essential for the developing brain. Bumetanide reduces GDP event frequency and hyperpolarizes neurons to decrease the depolarizing driving force for GABA.[40,41]

In conclusion, this study demonstrates that neonatal propofol and etomidate exposure enhances the GABAergic neurotransmission in pyramidal neurons by acting at presynaptic and/or postsynaptic sites, which involves the glutamatergic tonic drive. Bumetanide reverses the GABAergic neurotransmission alteration induced by neonatal propofol exposure.


## Acknowledgments

We would like to thank Dr. Anatoly E. Martynyuk who designed the research. We also thank Ms. Corey Astrom, Ms. Kathryn L. Bauman, and Ms. Poppy W. Meehan for their help in the manuscript preparation.

## Financial support and sponsorship

This work was supported by the grant from the National Institutes of Health (No. R01 GM93036-01A1).

## Conflicts of interest

There are no conflicts of interest.


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