Effect of thiopental sodium on N-methyl-D-aspartate-gated currents

[L'effet du thiopental sodique sur les récepteurs N-méthyl-D-aspartate]

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Purpose: N-methyl-D-aspartate (NMDA) receptors in the prefrontal cortex (PFC) are closely related with the excitability of pyramidal neurons and PFC function. As the effect of thiopental sodium on the central nervous system may partly result from the inhibition of PFC NMDA receptors, we investigated the effect of thiopental sodium with different concentrations on NMDA-gated currents in acutely dissociated rat PFC pyramidal neurons. We sought to determine whether thiopental sodium inhibits NMDA receptor function.

Methods: Three to four week old male Sprague-Dawley rats were sacrificed and the PFC was dissected. Pyramidal neurons from the PFC were prepared and standard whole-cell patch clamp recordings were performed. Escalating concentrations from 3–1000 μM NMDA were applied 100 μm from the pyramidal cells, and the concentration in the effect compartment related to 50% effect (EC50) of NMDA was determined for the ensuing experiments. One hundred μM NMDA alone (control) or NMDA with different concentrations (10–1000 μM) of thiopental sodium were applied. After the inhibitory concentration, in 50% of NMDA effect (IC50) of thiopental sodium was established this IC50 and NMDA 3–1000 μM were applied 100 μm from the pyramidal cells. The EC50 value of NMDA under the effect of IC50 thiopental sodium was determined.

Results: N-methyl-D-aspartate induced inward currents in a concentration-dependent manner, which were completely antagonized by 50 μM AP5. The maximal amplitude of NMDA-induced current was 1.15 ± 0.27 nA. The EC50 of NMDA was 53.6 ± 12.4 μM. The NMDA (100 μM)-gated current was inhibited by thiopental sodium in a concentration-dependent manner, and the IC50 of thiopental sodium was 33.6 ± 6.1 μM. Under the effect of 33.6 μM thiopental sodium, the maximal amplitude of NMDA-induced current was 0.87 ± 0.17 nA. The concentration-response curve of NMDA was shifted rightwards. The EC50 of NMDA was 128 ± 15 μM, which was greater than that of NMDA without thiopental sodium ($P < 0.01$).

Conclusions: Thiopental sodium decreases NMDA-gated currents in acutely dissociated rat prefrontal cortical pyramidal neurons in a concentration-dependent manner.

Objectif *: Les récepteurs de N-méthyl-D-aspartate (NMDA) dans le cortex préfrontal (CPF) sont en lien étroit avec l'excitabilité des neurones pyramidaux et la fonction du CPF. L'effet du thiopental sodique sur le système nerveux central pouvant résulter de l'inhibition des récepteurs de NMDA du CPF, nous recherchons l'effet de différentes concentrations de thiopental sodique sur les récepteurs NMDA dans des neurones pyramidaux très dissociés du CPF de rat. Nous voulons savoir si le thiopental sodique inhibe la fonction des récepteurs de NMDA.*

Méthode *: Des rats mâles Sprague-Dawley, de trois à quatre semaines, ont été sacrifiés et le CPF disséqué. Les neurones pyramidaux du CPF ont été préparés et des enregistrements standards selon la technique «patch clamp» ont été réalisés. Des concentrations croissantes de 3–1000 µM de NMDA ont été appliquées à 100 µm des cellules pyramidales et la concentration dans le compartiment effecteur, reliée à 50 % d'effet (EC50) de NMDA, a été déterminée pour les expériences ultérieures. Cent µM de NMDA seul (témoin) ou du NMDA avec différentes concentrations (10–1000 µM) de thiopental sodique ont été appliqués. Après l'établissement de la concentration inhibitrice de thiopental sodique chez 50 % d'effet de NMDA, cette IC50 et 3–1000 µM de NMDA ont été appliqués à 100 µm des cellules pyramidales. L'EC50 de NMDA sous l'effet de IC50 de thiopental sodique a été déterminé.*

Résultats *: Le N-méthyl-D-aspartate a induit des courants entrants, en fonction de la concentration, qui ont été complètement antagonisés par 50 µM d'AP5. L'amplitude maximale du courant induit par le NMDA a été de 1,15 ± 0,27 nA. L'EC50 du NMDA a été de 53,6 ± 12,4 µM. Le courant contrôlé par le NMDA (100 µM) a été inhibé par le thiopental sodique en fonction*

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de la concentration et l'IC50 du thiopental sodique a été de 33,6 ± 6,1 μM. Sous l'effet de 33,6 μM de thiopental sodique, l'amplitude maximale du courant induit par le NMDA a été de 0,87 ± 0,17 nA. La courbe de concentration-réponse du NMDA a été déplacée vers la droite. L'EC50 du NMDA a été de 128 ± 15 μM, ce qui est plus important que celui du NMDA sans le thiopental sodique (P < 0,01).

***Conclusion** : Le thiopental sodique a diminué, en fonction de la concentration, les courants contrôlés par le NMDA dans des neurones pyramidaux très dissociés de cortex préfrontal de rat.*

THE action of general anesthetics is considered to be mediated by inhibition of excitatory neural transmission and potentiation of inhibitory neural transmission.[1] It is known that glutamate and gamma-aminobutyric acid (GABA) are the most important excitatory and inhibitory neural transmitters in central nervous system.[2] In a previous study, pentobarbital potentiated $GABA_A$ receptors as a result of stabilizing the open states.[3] However, in another experiment, pentobarbital could elicit $GABA_A$ receptor currents at micromolar concentrations, and blocked $GABA_A$ receptor at higher concentrations.[4]

We know that $GABA_A$ receptors are not the only target for general anesthetics.[5] The NMDA receptor may also mediate the action of barbiturates. It has been reported that barbiturates decrease NMDA-gated currents in the spinal cord but have no effect on amino acid pathway gated currents.[6] In addition, pentobarbital has been shown to decrease NMDA receptor function in neurons isolated from rat olfactory brain by blocking the opening channel.[1]

In the central nervous system, the prefrontal cortex (PFC) plays a key role in the generation and modulation of consciousness,[7] which depends on rapid synaptic neural transmission mediated by the $GABA_A$ receptor, the NMDA receptor and other ionotropic receptors of glutamate and GABA.[8] It has been shown that NMDA-mediated channels are closed when unconsciousness occurs in status epilepticus.[9] As an *iv* general anesthetic, thiopental sodium induces unconsciousness and loss of memory. In this study, we investigate the effect of thiopental sodium with different concentrations (10–1000 μM) on NMDA-gated inwards currents in isolated pyramidal neurons from rat PFC, using whole-cell patch clamp techniques. We hypothesized that clinically relevant concentrations of thiopental sodium can depress NMDA receptor function in the PFC.

Materials and methods

All animal experiments followed the instructions for the care and use of animals by Tongji Medical College, Huazhong University of Science and Technology, and were approved by the Ethics Committee of Tongji Medical College.

Male Sprague-Dawley rats, three to four weeks old, weighing 120–150 g, were provided by the experimental animal centre of Tongji Medical College. Thiopental sodium was purchased from Shanghai Newasia Pharmacological Co. Ltd. (Certification 020702), NMDA and D(-)-2-amino-5-phosphonopentanoic acid (AP5, a NMDA receptor antagonist) were products of Sigma Co. (St. Louis, MO, USA). Other reagents were of analytical grade.

Preparation of prefrontal cortical slices and pyramidal neurons

The PFC pyramidal neurons were isolated as reported before.[10] Rats were decapitated, the PFC was removed (range: anterior to the bregma 2.2–3.5 mm, depth from the bregma 3–5 mm, lateral to the bregma 0.8–1.2 mm)[11] and rinsed in ice-cold 0.32 M sucrose solution. Two minutes later, the PFC was glued to the chilled stage of a vibratome and sliced to a thickness of 400 μm. These slices were then incubated in artificial cerebral spinal fluids for one to six hours at room temperature, which were composed of (in mM): NaCl 126.5, $NaHCO_3$ 27.5, KCl 2.4, KH_2PO_4 0.5, $CaCl_2$ 1.1, $MgCl_2$ 0.83, Na_2SO_4 0.5, and glucose 11.8, adjusted to pH 7.3, aerated with 95%O_2 and 5%CO_2, then incubated in oxygenated artificial cerebral spinal fluid (32°C) containing 1.1–1.4 $g{\cdot}L^{-1}$ protease for 30 min. The slices were transferred to a tube filled with artificial cerebral spinal fluid and subjected to mild trituration through heat polished pipettes of progressively smaller tip diameter in order to isolate single neurons, then transferred through a steel net to a 35-mm tissue culture dish.

Whole-cell patch clamp recordings

All electrophysiological studies were performed at room temperature (22°C) as reported before.[12–14] The isolated pyramidal neurons were allowed to settle to the bottom of the dish for 15 min before patch clamp recordings were made under an inverted microscope (Olympus IX70, Tokyo, Japan). The internal solution consisted of (in mM): KCl 150, $MgCl_2$ 2, HEPES 15, K-ATP 2, EGTA 5, Phosphocreatine 15, and creatine phosphokinase 50 $u{\cdot}mL^{-1}$ adjusted to pH 7.3 with 0.1M KOH. The external solution consisted of (in mM): NaCl 145, KCl 5, HEPES 10, $CaCl_2$ 2, glucose 10, TTX 0.001, and glycine 0.001,

adjusted to pH 7.4 with 0.1M NaOH. Patch clamp recordings were obtained from an EPC-9 patch clamp amplifier (HEKA, Lambrecht, Germany) that was monitored with an IBM personal computer running pulse 8.02 software. The patch recording electrodes were pulled from thin-walled borosilicate glass using a two-stage process from a vertical puller (PIP5, HEKA, Germany) to an electrode resistance of 3–6 MΩ in the water bath. When the cell membrane was ruptured and sealed (the magnitude of giga-seal > 1 GΩ), series resistance (6–10 MΩ) was compensated by 80% and monitored continually. When a significant increase of the series resistance (> 20%) occurred, the recordings were terminated. The cells were voltage-clamped at a holding membrane potential –60 mV. Data were filtered at10 kHZ and digitized at 25 kHZ with pulse 8.02 software, stored on the hard disk of the computer.

Drugs (NMDA or thiopental sodium) were prepared on the day of the patch recordings, and applied with a gravity-fed sewer pipe (Tongji Medical College, Wuhan, China). The internal diameter of the array was 100 μm. The external solution was changed by the SF-77B fast-step solution stimulus delivery device (Warner Instrument Co, Hamden, CT, USA) when the recordings on one cell were terminated. N-methyl-D-aspartate (3–1000 μM) was applied for eight sec from the array of the pipe 100 μm from the cells, the interval between applications was 1.5 min. [12] Next, 50 μM AP5 and 3–1000 μM NMDA were applied to confirm the inward currents mediated by NMDA receptors. The concentration in the effect compartment related to 50% effect (EC50) value of NMDA was achieved according to the Hill equation. In most neurons, the desensitization of NMDA-gated currents reached steady state after a four second application of NMDA. In this study; steady state NMDA-gated currents were considered the amplitude of currents at the end of eight second exposure of different concentrations of NMDA to confirm NMDA-gated currents reached steady state in all neurons. When the effect of thiopental sodium on NMDA-gated currents was studied, 100 μM NMDA alone (control) or NMDA with different concentrations of thiopental sodium was applied for eight seconds from the array of the pipe system 100 μm from the cells. The time interval between applications was 1.5 min. The concentrations of thiopental sodium applied were 10, 30, 100, 300, and 1000 μM, the inhibitory concentration in 50% of NMDA effect (IC50) value of thiopental sodium was achieved according to the Hill equation. Each experimental treatment was preceded and followed by application of NMDA alone as a control; if post-control value was less than 80% of pre-control value, the experimental data were deleted. Drug effects were expressed as the percent change of pre-control value. When the effect of IC50 thiopental sodium on the NMDA concentration-response curve was investigated, 3–1000 μM NMDA and IC50 thiopental sodium were applied 100 μm from the pyramidal cells. The application duration and the time interval were the same as mentioned above. The EC50 for NMDA under the effect of thiopental sodium at the IC50 was established.

Statistical analysis

The EC50 of NMDA was calculated according to the Hill equation: $I=I_{max}/[1+(EC50/ligand)^{nH}]$. The I_{max} value is the maximal response at the saturating concentration of NMDA, and nH is the Hill coefficient. The IC50 of thiopental sodium was achieved according to the Hill equation: $I=I_{max}/[1+(IC50/noncompetitive\ antagonist)^{nH}]$, where I_{max} is the maximal response at the saturating concentration of thiopental sodium, and nH is the Hill coefficient.[13]

One-way ANOVA followed by least squares analysis was performed to compare the responses between different concentrations of NMDA or the responses of NMDA under different concentrations of thiopental sodium. Chi-square tests were performed to compare the differences between the EC50 values of NMDA under the influence of thiopental sodium at the IC50 concentration and that of NMDA without thiopental sodium. Data are expressed as mean ± standard deviation. A P value < 0.05 was accepted as evidence of a significant difference.

Results

A total of 118 PFC pyramidal neurons were evaluated in this study, and data from eight cells were deleted due to "run-down" of the currents. Just one concentration or several concentrations of NMDA, thiopental sodium or NMDA with thiopental sodium were applied to a single neuron which depended on the excitability of the neuron in the experimental procedure.

N-methyl-D-aspartate-induced inward currents in PFC neurons

Data from 38 cells was recorded, and data from three cells was deleted due to the unknown phenomenon of the currents. In acutely dissociated pyramidal neurons, application of 3–1000 μM NMDA evoked inward currents in a concentration-dependent manner. These effects were completely blocked by the NMDA receptor antagonist AP5 with 50 μM (n = 11), confirm-

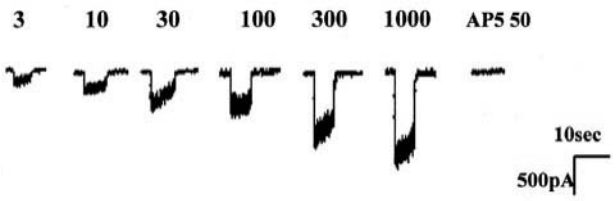

FIGURE 1 Representative whole-cell current responses of pyramidal neurons to different concentrations of N-methyl-D-aspartate (NMDA). The maximal amplitude of NMDA-induced current was 1.15 ± 0.27 nA (n = 11).

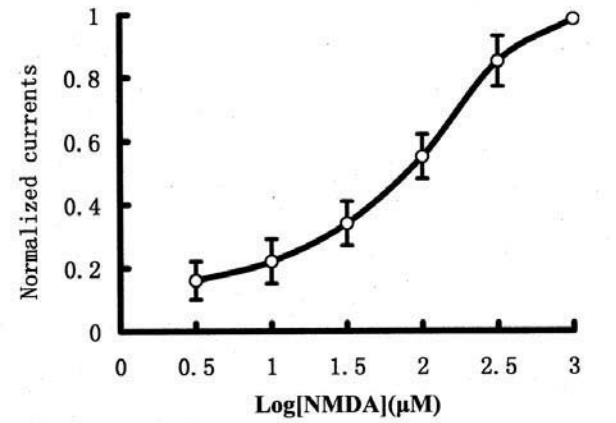

FIGURE 2 The concentration-response curve of N-methyl-D-aspartate (NMDA)-induced currents. The effect compartment related to 50% effect (EC50) value of NMDA was 53.6 ± 12.4 μM (n = 11).

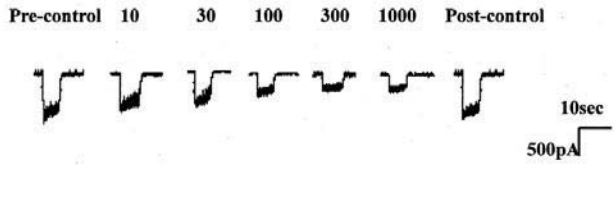

FIGURE 3 Representative whole-cell current response of a pyramidal neuron at a concentration of 100 μM N-methyl-D-aspartate (NMDA), alone or in combination with different concentrations of thiopental sodium. At pre-control, pyramidal neurons were exposed to 100 μM NMDA, before NMDA was combined with thiopental sodium. Post-control values reflect pyramidal neurons which were exposed to 100 μM NMDA after NMDA combined with thiopental sodium. The inhibitory concentration in 50% of NMDA effect (IC50) value of thiopental sodium was 33.6 ± 6.1 μM (n = 11).

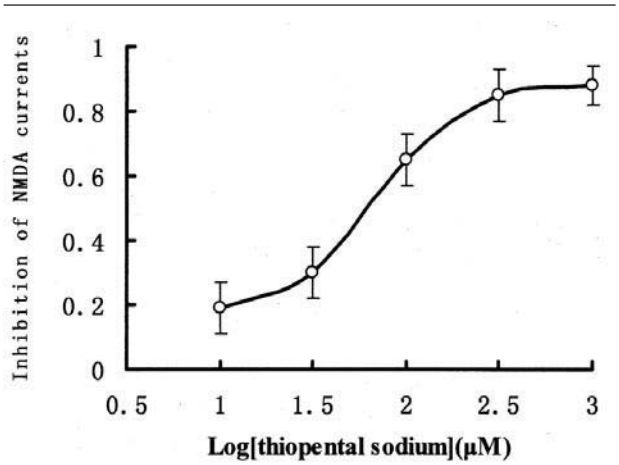

FIGURE 4 The concentration-response curve of thiopental sodium for inhibition of 100 μM N-methyl-D-aspartate (NMDA)-induced currents in PFC neurons. The maximal per cent inhibition of thiopental sodium was 88.3 ± 6.5% (n = 11). The inhibitory concentration in 50% of NMDA effect (IC50) of thiopental sodium was 33.6 ± 6.1 μM.

ing mediation by NMDA receptor (Figure 1). The concentration-response curve of NMDA is shown in Figure 2. The maximal amplitude of the NMDA current was 1.15 ± 0.27 nA. The EC50 value of NMDA was 53.6 ± 12.4 μM (n = 11).

Inhibitory effect of thiopental sodium on NMDA (100 μM)-induced inwards current in the PFC neurons

NMDA (100 μM) was applied at twice the EC50 value to induce a current with sufficient amplitude for inhibition of thiopental sodium. Thirty-one cells were recorded. When NMDA (100 μM) and thiopental sodium concentrations of 10, 30, 100, 300, or 1000 μM were applied, NMDA (100 μM)-induced current was inhibited by thiopental sodium in a concentration-dependent manner (n = 11, Figure 3). The maximal per cent inhibition of thiopental sodium was 88.3 ± 6.5% (n = 11). The concentration-response curve of thiopental sodium is shown in Figure 4. The IC50

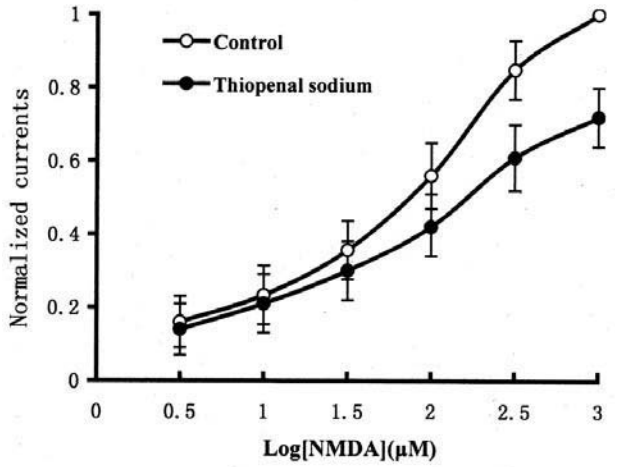

FIGURE 5 Concentration-response curves for N-methyl-D-aspartate (NMDA), (3–1000 μM) in the control (○) and in the presence of 33.6 μM thiopental sodium (●). N-methyl-D-aspartate concentration-response curve was shifted rightwards under the effect of 33.6 μM thiopental sodium. The maximal amplitude of the NMDA-induced current was 0.87 ± 0.17 nA in the presence of thiopental sodium at IC50 level. The effect compartment related to 50% effect (EC50) of NMDA increased from 53.6 ± 12.4 μM (control) to 128.3 ± 15.0 μM (thiopental sodium), ($P < 0.01$). $n = 11$ neurons for each concentration of NMDA.

value of thiopental sodium was 33.6 ± 6.1 μM.

Effects of thiopental sodium with IC50 level on NMDA-induced currents in the PFC neurons

Forty-nine cells were recorded; data from five cells were deleted due to the unkown phenomenon of the currents. The IC50 of thiopental sodium (33.6 μM) and NMDA 3–1000 μM were applied to the PFC neurons. Under the effect of 33.6 μM thiopental sodium, NMDA evoked inward currents in a concentration-dependent manner; the maximal amplitude of NMDA-induced current was 0.87 ± 0.17 nA. The concentration-response curve of NMDA was shifted rightwards. The EC50 of NMDA was 128 ± 15 μM, which was significantly higher than that of NMDA without the effect of thiopental sodium (control), ($P < 0.01$, $n = 11$, Figure 5).

Discussion

In mammals, pyramidal neurons are the main excitatory cells in the PFC, which account for 70% of all neurons.[15] It has been reported that the excitability of the PFC and the excitatory output from the PFC are predominantly dependent on NMDA receptor function.[16] In this study, thiopental sodium decreased NMDA-gated currents in the PFC pyramidal neurons, which may decrease the excitability of the PFC.

There may be various mechanisms involved in the inhibitory effect of thiopental sodium on NMDA-gated currents. First, thiopental sodium may decrease the fluidity of the pyramidal neural sarcolemma, so as to decrease NMDA receptor function. A previous study showed that increasing the percentage of cholesterol component in neural sarcolemma could facilitate the sensitivity of $GABA_A$ receptors to propofol.[17] Second, $GABA_A$ receptors may mediate the inhibitory effect of thiopental sodium. Gamma-aminobutyric $acid_A$ receptors also exist in neural sarcolemma of pyramidal cells,[18] and thiopental sodium may directly activate $GABA_A$ receptors to induce hyperpolarization of pyramidal neurons to decrease NMDA-gated currents. Third, thiopental sodium may interfere with NMDA receptor channels directly. It has been reported that the NMDA receptor specific antagonist MK801 blocked NMDA receptor channels at the inner part, and had a selective binding to the opening mode.[19] Ketamine (an NMDA receptor nonspecific antagonist) decreases the opening frequency and duration of NMDA receptor channels in a concentration-dependent manner.[20] Accordingly, thiopental sodium may possibly decrease the opening frequency and duration of NMDA receptor channels. Furthermore, it has been shown that NMDA receptor channels are modulated by protein kinase and protein phosphatases.[21,22] The inhibitory effect of thiopental sodium on NMDA-gated currents may also result, in part, from inhibition on protein kinase or phosphatases.

When NMDA receptors are activated in the PFC, Ca^{2+} flux into the pyramidal cells, eventually modulate the excitability of the PFC, through a series of intracellular reactions, such as activation of Ca^{2+}/calmodulin-dependent protein kinase II.[9] In addition, Ca^{2+} entry can also activate nitric oxide synthase and nitric oxide is produced. It has been reported that thiopental sodium 30 $mg \cdot kg^{-1}$ intraperitoneally decreased nitric oxide synthase activity and nitric oxide production in an animal model.[23]

In this study, acutely dissociated PFC pyramidal neurons were used to record NMDA-gated currents, which were not interrupted by neural transmission in this brain region. It is unclear whether thiopental sodium would have the same effect on PFC NMDA-gated currents in a whole animal model. It has been reported that the EC50 of thiopental sodium for general anesthesia in the whole animal is 25 μM (free

drug concentration),[24] but in this study, the IC50 of thiopental sodium for NMDA-gated currents in isolated pyramidal neurons was 33.6 μM, about 35% more than the EC50 in the whole animal. This may result from the differences between *in vivo* and *in vitro* experiments. Another limitation is that in this study, patch recordings were performed at room temperature (22°C). While temperature may have affected NMDA-mediated currents in isolated neurons, in this experimental procedure, neurons could not maintain their excellent excitability above room temperature. Results from previous studies[12–14] and our own, show that the ligand-gated currents were stable just at room temperature. In a preliminary experiment, we tried to record NMDA-gated currents at 37°C, but the isolated PFC neurons had limited excitability and were prone to collapse at that temperature.

We know now the central nervous system mechanisms of thiopental sodium are very complex, and that a presynaptic effect may be involved. It was reported in our previous work that clinically relevant concentrations of thiopental sodium can decrease glutamate release from rat prefrontal cortical synaptosomes, without affecting GABA release.[25] In addition, $GABA_A$ receptor function in the PFC may be potentiated by thiopental sodium. Alternatively, thiopental sodium may affect neural transmission mediated via other transmitters such as acetylcholine, adenosine diphosphate and 5-hydroxytryoptamine in the PFC and in the other brain regions. Such possibilities will be the subject of further investigations, as we expand our understanding of anesthetic mechanisms

In conclusion, we have shown that thiopental sodium decreases NMDA-gated currents in acutely dissociated rat PFC pyramidal neurons in a concentration-dependent manner.

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