Blockade of Glutamate Receptors and Barbiturate Anesthesia

Increased Sensitivity to Pentobarbital-induced Anesthesia Despite Reduced Inhibition of AMPA Receptors in GluR2 Null Mutant Mice

Daisy T. Joo, M.D.,* Zhigang Xiong, Ph.D.,† John F. MacDonald, Ph.D.,‡ Zhengping Jia, Ph.D.,§ John Roder, Ph.D.,∥ Jim Sonner, M.D.,# Beverley A. Orser, M.D., Ph.D., F.R.C.P.C.**

Background: Barbiturates enhance γ-aminobutyric acid type A (GABA_{A}) receptor function and also inhibit the α-aminoo-3-hydroxy-5-methyl-4-oxazolepropionic acid (AMPA) subtype of glutamate receptor. The relative contribution of these actions to the behavioral properties of barbiturates is not certain. Because AMPA receptor complexes that lack the GluR2 subunit are relatively insensitive to pentobarbital inhibition, GluR2 null mutant mice provide a novel tool to investigate the importance of AMPA receptor inhibition to the anesthetic effects of barbiturates.

Methods: GluR2 null allele (-/-), heterozygous (+/-), and wild-type (+/+) mice were injected with pentobarbital (30 and 35 mg/kg intraperitoneally). Sensitivity to anesthetics was assessed by measuring the latency to loss of righting reflex, sleep time, and the loss of corneal, pinel, and toe-pinch withdrawal reflexes. In addition, patch-clamp recordings of acutely dissociated CA1 hippocampal pyramidal neurons from (-/-) and (+/+) mice were undertaken to investigate the effects of barbiturates on kainate-activated AMPA receptors and GABA-activated GABA_A receptors.

Results: Behavioral tests indicate that sensitivity to pentobarbital was increased in (-/-) mice. In contrast, AMPA receptors from (-/-) neurons were less sensitive to inhibition by pentobarbital (concentrations that produced 50% of the maximal inhibition [IC_{50}], 301 vs. 51 μM, thiopental IC_{50} 155 vs. 34 μM), and phenobarbital IC_{50} 930 vs. 205 μM) compared with wild-type controls, respectively. In addition, the potency of kainate was greater in (-/-) neurons, whereas no differences were observed for the potentiation of GABA_A receptors by pentobarbital.

Conclusions: The GluR2 null mutant mice were more sensitive to pentobarbital anesthesia despite a reduced sensitivity of GluR2-deficient AMPA receptors to barbiturate blockade. Our results indicate that the inhibition of AMPA receptors does not correlate with the anesthetic effects of barbiturates in this animal model. We postulate that the increase in the sensitivity to anesthetics results from a global suppression of excitatory neurotransmission in GluR2-deficient mice. (Key words: Electrophysiology; knockout; righting reflex; voltage clamp.)

NORMAL neuronal transmission depends on the delicate balance between excitatory and inhibitory synaptic inputs. General anesthetics, including barbiturates, are thought to depress neuronal function by disrupting this interplay. Barbiturates enhance the activity of γ-aminobutyric acid (GABA)ergic synapses and reduce glutamatergic transmission.
tyrctrone A (GABA<sub>A</sub>) receptors, thereby facilitating inhibitory postsynaptic currents. Barbiturates also inhibit the "-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of glutamate receptors at clinically relevant concentrations. Although the relative importance of glutamate receptors to the anesthetic effects of barbiturates is not certain, the anticonvulsant<sup>2</sup> and neuroprotective<sup>3</sup> effects of barbiturates have been attributed, in part, to the inhibition of AMPA receptors.

Glutamate is the major excitatory neurotransmitter in the central nervous system, and the AMPA subtype of glutamate receptor mediates the fast component of excitatory postsynaptic currents.<sup>4,5</sup> AMPA receptors consist of protein subunits that assemble into pentameric<sup>6</sup> or tetrameric<sup>7,8</sup> structures that contain an intrinsic channel pore. Based on sequence homology, four subunit genes have been identified and are referred to as GluR1 to GluR4.<sup>9</sup> The expression of these genes and the splicing and editing of their pre-mRNA products<sup>10,11</sup> vary in different populations of neurons, resulting in a diversity of AMPA receptors.<sup>12</sup>

The physiologic and pharmacologic properties of native and recombinant AMPA receptors critically depend on the subunit composition. For example, the presence of the GluR2 subunit confers a ninefold to 30-fold<sup>13,14</sup> decrease in calcium (Ca<sup>2+</sup>) permeability to AMPA receptors.<sup>15</sup> This reduced Ca<sup>2+</sup> permeability is attributed to a positively charged arginine (R) residue in the membrane reentrant pore loop (M2) of the GluR2 subunit. The gene for the GluR2 subunit codes for an uncharged glutamine (Q); however, the codon is "edited" at the pre-mRNA stage to one encoding arginine at the "Q/R edited site" in the 586 position of M2.<sup>11,16</sup> The other AMPA receptor subunits (GluR1, GluR3, and GluR4) are unedited and contain a neutral glutamine residue at this site.

AMPA receptors that contain the GluR2 subunit are further characterized by a reduced sensitivity to endogenous and exogenous polyamines<sup>17,18</sup> and an increased sensitivity to barbiturates.<sup>19,20</sup> The resistance of GluR2-containing receptors to polyamines is attributed to the charged arginine residue at the Q/R edited site that repels the positive charge of polyamines. The mechanism underlying the altered sensitivity of GluR2-containing receptors to barbiturates is less certain because barbiturates are relatively uncharged at physiologic pH. Nevertheless, recombinant receptors that lack the GluR2 subunit<sup>19</sup> or its Q/R edited site<sup>20</sup> demonstrate a fivefold to 10-fold reduced sensitivity to pentobarbital, respectively. These results predict that neurons deficient of the GluR2 subunit would be less sensitive to barbiturate inhibition.

Null mutant mice, deficient of the GluR2 subunit, were recently developed by Jia et al.<sup>13</sup> Gene targeting in embryonic stem cells allowed the disruption of the M1 and pore loop regions of the GluR2 gene, preventing the expression of the entire GluR2 subunit. GluR2 null allele mutants are fertile but show poor parenting ability, are smaller during early development, and have a higher postnatal mortality. However, their weight and size are similar to normal littermates by 5 to 7 weeks, and, subsequently, they have a normal life expectancy.<sup>13</sup> Adult GluR2 null mutant mice demonstrate decreased exploratory behaviors and impaired motor coordination.<sup>13</sup> Nevertheless, they demonstrate a brisk righting reflex and no differences in corneal, pineal, or toe-pinchoff withdrawal reflexes under control conditions.

GluR2 null allele mutant mice provide an experimental model to study the correlation between the in vivo sensitivity to barbiturates and blockade of AMPA receptors in vitro. We tested the hypothesis that GluR2-deficient mice demonstrate a decreased sensitivity to the anesthetic effects of barbiturates compared with wild-type littersmates because of the reduced blockade of AMPA receptors.

Materials and Methods

Behavioral Study of Anesthetic Sensitivity

The sensitivity to pentobarbital was investigated in 28 GluR2 null mutant (+/−), 45 wild-type (+/+), and 26 heterozygous (+/−) littermate mice older than 6 weeks of age. Pentobarbital 30 mg/kg (Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, Canada; 65 mg/ml diluted to 1 mg/ml in 0.9% normal saline) was injected intraperitoneally after an aspiration test. The mice were then placed in a plastic container that was warmed by a heat lamp. An investigator who was blinded to the genotype observed the mice and recorded the time interval between the injection of pentobarbital and the loss of righting reflex (LORR). At 2-min intervals, the animals were gently placed on their backs, and if they failed to right themselves, the time was noted. After the LORR, supplemental oxygen (4 l/min) was delivered through a nose cone placed near the snout. Mice that failed to lose their righting reflex were observed for at least 60 min. In addition, the corneal reflex was tested at 2-min intervals by lightly brushing the cornea with a 1.0 proline suture. The pineal reflex was tested using the suture to irritate...
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the lower aspect of the ear helix, and toe-pinch withdrawal was assessed by squeezing a hindlimb toe using a rubber-shod hemostat. Respiratory rate was measured during a 15-s interval every 2 min. Respiratory depression was defined as a \( \geq 25\% \) decrease in baseline respiratory rate determined before the injection. Finally, the time to the recovery of the righting reflex, or sleep time, was noted, and mice were observed for an additional 20 min before being returned to their cages. Animals were maintained under the heat lamp during the observation period. Two weeks to 1 month later, 15 (+/+) and 14 (−/−) mice from this initial group were injected with pentobarbital 35 mg/kg intraperitoneally and observed according to the protocol described.

In eight additional animals, the body temperature was measured by inserting a rectal probe (Fisher Scientific, Hampton, NH) immediately after pentobarbital 30 mg/kg was administered intraperitoneally. Temperature was measured at 2-min intervals until the animals regained the righting reflex or until 15 min had elapsed for mice that did not lose this reflex.

All animal study protocols were approved by the animal facility at the Samuel Lunenfeld Research Centre, Mount Sinai Hospital, Toronto, and the Animal Care Committee of the University of Toronto.

**Plasma Pentobarbital Levels**

The plasma concentration of pentobarbital was measured in six subjects consisting of three (+/+) mice and three (−/−) mice that had not been previously injected with the barbiturate. Animals were killed by decapitation 5 min after the injection of pentobarbital (30 mg/kg intraperitoneally). The sample was obtained at 5 min because this latency to LORR in the (−/−) mice. Approximately 1 ml blood was collected from the decapitation site into a 1.5-ml microcentrifuge tube. Samples were centrifuged, and pentobarbital concentrations were assayed from the plasma using high-performance liquid chromatography.

**Acute Isolation of Hippocampal Neurons**

Mice older than 6 weeks of age were anesthetized with halothane and then decapitated. The entire brain was then rapidly removed and rinsed in cold (4°C) extracellular solution consisting of (in mM): NaCl 140, CaCl\(_2\) 1.3, KCl 5.4, N-2-hydroxy-ethylpiperazine-N’-2-ethanesulphonic acid (HEPES) 25, glucose 33, and sodium chloride 2, CaCl\(_2\) 1, magnesium adenosine triphosphate 4. This solution was buffered to a pH of 7.4 using NaOH, and the osmolality was adjusted to 300–310 mOsm. For the experiments in which GABA was applied so that a detectable outward current could be recorded after the application of GABA.

Acutely isolated neurons were voltage clamped at a holding potential of −60 mV. Whole-cell currents were recorded using the Axopatch 1D amplifier (Axon Instruments Inc., Foster City, CA) and data were filtered (2 kHz), digitized, and acquired on-line using the pCLAMP5 program (Axon Instruments). Changes in series resistance were monitored during the recordings by measuring the capacitance transient resulting from a hyperpolarizing test pulse of 10 mV. Recordings that demonstrated marked changes in access resistance were not used for data analysis.
A multibarrel perfusion system was used to achieve a rapid exchange (30 ms) of the extracellular solution. Three square capillary tubes (400 μm × 400 μm) were glued together and mounted on a Leitz manipulator (Wild Leitz Canada Ltd., Midland, Ontario, Canada). Each barrel was connected to a 7-ml reservoir of perfusion fluid, and the flow rate set to approximately 0.5 ml/min by adjusting the height of the reservoir. A computer-driven motor-based stepper (Vexta motor; Oriental Motor Co., Torrance, CA) was used to laterally move the capillary assembly. After the formation of the whole-cell configuration, neurons were lifted into the outflow of the barrels. Cells were perfused with the extracellular solution or solutions containing the AMPA receptor agonist, kainate (Sigma Chemical Co.), GABA (Sigma Chemical Co.), or barbiturates (pentobarbital, thiopental, and phenobarbital).

Glutamate-evoked currents mediated by AMPA receptors rapidly desensitize to a low steady-state amplitude. Although kainate-evoked currents also desensitize, the extent of desensitization is considerably less, and the steady-state current is greater compared with glutamate-evoked responses. Therefore, kainate was used to investigate the inhibition of AMPA receptors by barbiturates as previously described. Various concentrations of barbiturates were applied during the steady-state response evoked by kainate. It is unlikely that the activation of kainate receptors, which undergo a fast pronounced desensitization, contributes appreciably to the currents recorded in these experiments. Because it is the endogenous ligand of AMPA receptors, glutamate (5 mM) was applied to activate AMPA receptors in another set of experiments. This saturating concentration of glutamate was applied with the N-methyl-D-aspartate receptor antagonist, dl-2-amino-5-phosphonovaleric acid (40 μM; Sigma Chemical Co.), and MgCl₂ 2 mM in the absence and presence of pentobarbital 30 μM and 100 μM. For these experiments, bicuculline 20 μM was added to all solutions to inhibit barbiturate activated Cl⁻ current mediated by GABAₐ receptors.

The current amplitude was measured using pCLAMP (Axon Instruments), and data were plotted using GraphPad Prism (Graph Pad, San Diego, CA). Concentration-response relationships for kainate-evoked currents and GABA-evoked currents recorded in the presence and absence of pentobarbital 30 μM were fitted using a modified version of the Michaelis-Menten equation by the least squares method. The concentration of kainate that produced 50% of the maximal response (EC₅₀) and the Hill coefficient (nₘ) were determined according to the equation:

\[ I = I_{\text{max}} \times \frac{1}{1 + \left( \frac{\text{EC}_{50}}{[\text{ligand}]} \right)^{n_{\text{H}}}} \]

where \( I_{\text{max}} \) is the maximal response observed at a saturating concentration of the agonist. Membrane capacitance, as an approximation of neuron size, was estimated from the area under the capacitance transient evoked by a 10-mV hyperpolarizing pulse applied after the formation of the whole-cell configuration.

For concentration-inhibition analysis, kainate was applied at the EC₅₀ concentration in the absence or presence of the barbiturates. The concentrations of pentobarbital, thiopental, or phenobarbital that produced 50% of the maximal inhibition (IC₅₀) were determined from the concentration-inhibition curves. Data points were normalized to the maximal inhibition and fit according to the equation:

\[ I = I_{\text{max}} \times \frac{1}{1 + \left( \frac{\text{IC}_{50}}{[\text{ligand}]} \right)^{n_{\text{H}}}} \]

where \( I_{\text{max}} \) is the maximal inhibition produced by a saturating concentration of the antagonist.

GABA and glutamate responses recorded in the presence of pentobarbital were examined for changes in amplitude of the peak and steady-state currents, respectively.

**Statistical Analysis**

All results were reported as mean ± SEM unless otherwise indicated. For the behavioral study, the latency to LORR and sleep time were compared between groups using a one-way analysis of variance (SigmaStat 2.0 software; SPSS Inc., San Rafael, CA). The chi-square and Fisher exact tests were used to compare the number of mice in each group that showed the loss of the righting, corneal, pinéal, and toe-pinch withdrawal reflexes. Where appropriate, the EC₅₀ values for kainate and GABA-evoked currents, as well as the IC₅₀ values for pentobarbital, thiopental, and phenobarbital for (+/+) and (-/-) neurons were compared using the Student t test. Data sets for which tests of normality failed were analyzed with corresponding nonparametric tests.

**Results**

**Anaesthetic Sensitivity to Pentobarbital Increased in GluR2 Null Mutant Mice**

The study population of (+/+) and (-/-) mice were initially organized into three groups according to post-
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Table 1. Demographics of Behavioral Study

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (+/+) (n = 28)</th>
<th>Group 2 (+/-) (n = 8)</th>
<th>Group 3 (-/-) (n = 9)</th>
<th>Group 1 (+/+) (n = 26)</th>
<th>Group 1 (+/-) (n = 13)</th>
<th>Group 2 (-/-) (n = 8)</th>
<th>Group 3 (-/-) (n = 7)</th>
</tr>
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<tbody>
<tr>
<td>Age (wk)</td>
<td>7.9 ± 0.3</td>
<td>24.3 ± 0.6</td>
<td>54.6 ± 1.4</td>
<td>7.7 ± 0.3</td>
<td>9.4 ± 0.5</td>
<td>22.8 ± 0.1</td>
<td>55.8 ± 1.8</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>28.7 ± 0.9</td>
<td>35.1 ± 2.4</td>
<td>35.6 ± 1.6</td>
<td>29.2 ± 0.9</td>
<td>27.9 ± 0.7</td>
<td>31.7 ± 0.6</td>
<td>35.8 ± 2.5</td>
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Table 2. Behavioral Study Results after Pentobarbital 30 mg/kg Intraperitoneally

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<thead>
<tr>
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<th>+/-</th>
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<tr>
<td></td>
<td>(n = 45)</td>
<td>(n = 26)</td>
<td>(n = 28)</td>
</tr>
<tr>
<td>Number (%) of mice with loss of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>righting reflex</td>
<td>25 (56)</td>
<td>17 (65)</td>
<td>26 (93)*</td>
</tr>
<tr>
<td>Number (%) of mice with loss of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>corneal reflex</td>
<td>28 (62)</td>
<td>17 (65)</td>
<td>25 (89)*</td>
</tr>
<tr>
<td>Number (%) of mice with loss of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pineal reflex</td>
<td>8 (18)</td>
<td>10 (38)</td>
<td>18 (64)*</td>
</tr>
<tr>
<td>Number (%) of mice with respiratory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>depression</td>
<td>27 (60)</td>
<td>15 (58)</td>
<td>17 (61)</td>
</tr>
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* Significantly different from (+/-) mice (P < 0.05, using the chi-square test).

After intraperitoneal injection of pentobarbital 30 mg/kg, a greater proportion of the (-/-) mice demonstrated LORR (table 2) and a shorter latency to LORR (5.1 ± 0.5 min, n = 26; 6.5 ± 0.4 min, n = 17; 7.8 ± 0.9, n = 25; P < 0.05) compared with (+/-) or (+/+) littermates, respectively (fig. 1A). GluR2 null mutant mice also demonstrated a longer sleep time (18.1 ± 1.5 vs. 10.3 ± 1.3 and 10.8 ± 1.5 min; P < 0.05; fig. 1B). A greater proportion of (-/-) mice lost their corneal and pineal reflexes compared with the (+/+), and (+/-) mice (P < 0.05; table 2). Loss of the toe-pinch withdrawal reflex was not demonstrated in any of the mice after injection of pentobarbital. The enhanced sensitivity to pentobarbital in (-/-) mice was not likely attributed to pharmacokinetic factors because the plasma concentration of pentobarbital was similar, at least at the 5-min interval, in (-/-) mice and (+/-) littermates (128.8 ± 15.9 μM, n = 3 vs. 113.3 ± 12.8 μM, n = 3, respectively; P > 0.05).

Some of the mice that were not killed for the electrophysiologic studies were allowed to recover for a minimum 2-week period. Pentobarbital 35 mg/kg was administered intraperitoneally as previously described. Three mice, two (-/-) and one (+/+), died from respiratory failure, whereas 14 (+/+) and 12 (-/-) mice were successfully anesthetized, and most of the subjects demonstrated LORR (table 3). Consistent with our previous findings, the (-/-) mice showed a decreased time to LORR (4.1 ± 0.4 min, n = 12 vs. 5.7 ± 0.3 min, n = 13; P < 0.05) and increased sleep time (24.5 ± 3.5 min, n = 12 vs. 12.6 ± 2.8 min, n = 12; P < 0.05). A greater proportion of (-/-) mice lost their pineal and corneal reflexes compared with (+/+) littermates; however, these differences were not statistically significant (table 3). The incidence of respiratory depression was not different between the (+/+) and (-/-) mice (tables 2 and 3).

Because the sensitivity to anesthetics can be influenced by core temperature, eight additional mice were observed for changes in rectal temperature after pentobarbital 30 mg/kg was administered intraperitoneally. No significant differences in temperature was detected at any time after the pentobarbital injection, including at 4 min [37.0 ± 0.4°C (+/+), n = 4 vs. 36.5 ± 0.4°C (-/-), n = 4] and 10 min [37.3 ± 0.2°C (+/+), n = 4 vs. 36.8 ± 0.3°C (-/-), n = 4].

In summary, these results demonstrate an increased anesthetic sensitivity to pentobarbital in the (-/-) mice compared with (+/-) and (+/+) littermates as evidence by a shorter latency to LORR, longer sleep time, and the greater proportion of mice that lost the corneal and pineal reflexes. This enhanced sensitivity could not be attributed to differences in blood concentrations or changes in rectal temperature.

**Potency of Kainate Is Increased in GluR2-Deficient AMPA Receptors**

The subunit composition of ligand-gated receptors influences the EC_{50} value of the receptor for agonist, as well as their sensitivity to pharmacologic agents. Furthermore, the extent of inhibition produced by an antagonist can vary with the concentration of agonist. Therefore, to ensure that equi-effective concentrations of agonist were used to elicit control currents in (-/-) mice.

Anesthesiology, V 91, No 5, Nov 1999

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and (+/+) neurons, the concentration-response relationships for kainate-evoked currents were first examined.

Applications of kainate (> 10 μM) activated an inward current in all hippocampal neurons tested. The maximum current evoked by a saturating concentration of kainate (I_max) was not influenced by the presence of the GluR2 subunit: (-/-)I_max = 1,341 ± 217 pA, n = 15 versus (+/+)I_max = 1,746 ± 233 pA, n = 12 (P > 0.05). Similarly, no differences were observed in the membrane capacitance in the dissociated cells: (-/-) 14.2 ± 3.9 pF versus (+/+) 14.9 ± 3.8 pF. The concentration-response relationship indicated the EC_{50} value for kainate-evoked current was significantly lower for (-/-) neurons compared with (+/+) neurons (136 ± 7 μM, n = 17 vs. 226 ± 23 μM, n = 15; P < 0.05), respectively. Our results are consistent with previous reports of a higher potency for kainate in recombinant AMPA receptors that lack the GluR2 subunit.20 The EC_{50} values for receptors containing the GluR2_{pip} subunit in combination with GluR1 (EC_{50} = 110 μM) or GluR1/GluR3_{pip} (EC_{50}, 100 μM) were greater than those values for homomeric GluR1 (EC_{50}, 32 μM) or dimeric GluR1/GluR3_{pip} (EC_{50}, 55 μM) receptors. The EC_{50} value we report for (+/+) neurons (EC_{50}, 226 μM) approximate those values reported for pyramidal neurons isolated from the CA3 region (EC_{50}, 341 μM) or CA1 region (EC_{50}, 474 μM) of the rat hippocampus.27 In addition, the Hill coefficient (1.69 ± 0.04 vs. 2.06 ± 0.13; P < 0.05) was lower for (-/-) neurons compared with (+/+) neurons, respectively (fig. 2), suggesting a decreased cooperativity for kainate activation of GluR2-deficient AMPA receptors.28

Table 3. Behavioral Study Results after Pentobarbital 35 mg/kg Intraperitoneally

<table>
<thead>
<tr>
<th></th>
<th>+/+ (n = 14)</th>
<th>-/- (n = 12)</th>
</tr>
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<tbody>
<tr>
<td>Number (%) of mice with loss of righting reflex</td>
<td>13 (93)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>Number (%) of mice with loss of corneal reflex</td>
<td>11 (79)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>Number (%) of mice with loss of pineal reflex</td>
<td>3 (21)</td>
<td>7 (68)</td>
</tr>
<tr>
<td>Number (%) of mice with respiratory depression</td>
<td>12 (86)</td>
<td>8 (67)</td>
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</table>
Fig. 2. Kainate-activated currents in acutely isolated hippocampal pyramidal neurons from wild-type (+/+)) and mutant (−/−) mice. (A) Representative kainate-evoked currents at subsaturating (30, 100, and 300 μM) and saturating (1000 μM) concentrations are shown. (B) Concentration-response curves for kainate-evoked currents in (+/+) neurons (squares), and (−/−) neurons (triangles) were constructed by normalizing to the maximal response and fitting the data points using the modified Michaelis-Menten equation. The concentrations that activated 50% of the maximal current (EC50) and Hill coefficients (nH) were: (+/+) EC50 226 ± 23 μM (n = 15); (−/−) EC50 136 ± 7 μM (n = 17); (+/+) nH 2.06 ± 0.13; and (−/−) nH 1.69 ± 0.04.

Barbital inhibition was approximately sixfold greater for (−/−) neurons compared with (+/+) neurons (301 ± 52 μM vs. 51 ± 10 μM; P < 0.05). Similarly, the IC50 values for thiopental (153 ± 29 μM vs. 54 ± 6 μM; P < 0.05) and phenobarbital (930 ± 344 μM vs. 205 ± 55 μM; P < 0.05) were fourfold to fivefold greater in (−/−) compared with (+/+) neurons, respectively (Figs. 4B and 4C). These results demonstrate a reduced barbiturate sensitivity of AMPA receptors in GluR2-deficient neurons. No significant differences in the Hill coefficient (nH) were observed for pentobarbital IC50 ([+/+] nH = 1.00 ± 0.03 and [−/−] nH = 1.31 ± 0.27] and thiopental ([+/+] nH = 1.08 ± 0.05 and [−/−] nH = 0.98 ± 0.16]. However, the Hill coefficient for phenobarbital was increased in the (−/−) neurons: ([+/+] nH = 1.12 ± 0.07 and [−/−] nH = 1.63 ± 0.18 (P < 0.05). The reduction in the slope factor for barbiturate concentration-inhibition curves reported for recombinant GluR2-deficient receptors was not demonstrated in (−/−) neurons.

Consistent with these results, the steady-state current evoked by glutamate (5 mM) was also sensitive to inhib-
A (+/+)

B (-/-)

<table>
<thead>
<tr>
<th>Pentobarbital</th>
<th>Kainate</th>
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<tbody>
<tr>
<td>0 µM</td>
<td>30 µM</td>
</tr>
<tr>
<td>100 µM</td>
<td>300 µM</td>
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<tr>
<td>1000 µM</td>
<td>3000 µM</td>
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1000 pA | 2000 ms

400 pA | 2000 ms

Fig. 3. Inhibition of kainate-evoked currents by pentobarbital. The application of various concentrations of pentobarbital (0, 30, 100, 300, 1,000, and 3,000 µM) are shown for current activated by 300 µM kainate in (A) wild-type (+/+) neurons and by 150 µM kainate in (B) mutant (-/-) neurons. Kainate was applied as indicated by the solid bar before, during, and after the application of pentobarbital.

Inhibition by pentobarbital. Pentobarbital 30 µM and 100 µM caused a significant inhibition of the current recorded from (+/+ ) neurons (30% ± 7% and 49% ± 6%, respectively; n = 11; P < 0.05) but not from (−/−) neurons (8% ± 11% and 24% ± 12%; n = 4; P = 0.40 and 0.26, respectively). Therefore, a decrease in barbiturate inhibition of AMPA receptors in (−/−) neurons was also demonstrated when the endogenous ligand, glutamate, was used to activate AMPA receptors.

**Potentiation of GABA<sub>A</sub> Receptors by Barbiturates is Unchanged in GluR2-deficient Neurons**

The GABA<sub>A</sub> receptor is thought to be a primary site of action of barbiturates. Therefore, we also investigated the effects of pentobarbital (30 µM) on GABA<sub>A</sub> receptor-mediated current activated by a subsaturating (30 µM) and a saturating concentration of GABA (600 µM). No differences were observed in the maximal amplitude of currents evoked by GABA 30 µM recorded from (−/−) neurons (1,489 ± 345 pA, n = 10) or (+/+ ) neurons (1,160 ± 235 pA, n = 13), respectively. Similarly, no difference in the maximal current activated by GABA 600 µM was apparent for responses from (−/−) neurons (4,654 ± 612 pA, n = 12) and (+/+ ) neurons (4,205 ± 695 pA, n = 12). Pentobarbital (30 µM) applied in the absence of GABA did not activate inward current in any of the neurons. However, pentobarbital (30 µM) potentiated the peak currents evoked by 30 µM GABA but not 600 µM GABA (fig. 5). The effects of pentobarbital on the GABA concentration-response relationship were also investigated. The calculated EC<sub>50</sub> values for GABA were: (−/−), 51 ± 6 µM (n = 17); and (+/+ ), 46 ± 6 µM (n =
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Fig. 4. Concentration–inhibition curves for barbiturate modulation of AMPA receptors in mutant (−/−) and wild-type (+/+) neurons. The concentrations ranged from 10 to 3,000 μM of (A) pentobarbital, (B) thiopental, and (C) phenobarbital in (+/+) (squares) and (−/−) (triangles) neurons. The IC₅₀ values for each barbiturate and Hill coefficients (nₑ) were determined by fitting the curves to a standard Hill equation. Pentobarbital IC₅₀: (+/+) 51 ± 10 μM (n = 7); (−/−), 301 ± 52 μM (n = 10); nₑ: (+/+) 1.00 ± 0.03; (−/−), 1.31 ± 0.27. Thiopental IC₅₀: (+/+) 34 ± 5 μM (n = 6); (−/−), 155 ± 29 μM (n = 5); nₑ: (+/+) 1.08 ± 0.05; (−/−), 0.98 ± 0.16. Phenobarbital IC₅₀: (+/+) 205 ± 55 μM (n = 7); (−/−), 930 ± 130 μM (n = 7); nₑ: (+/+) 1.12 ± 0.07; (−/−), 1.63 ± 0.18.

13). Pentobarbital caused a shift to the left of the GABA concentration–response curve and significantly reduced the GABA EC₅₀ value to 43 ± 6 μM (n = 11) and 34 ± 5 μM (n = 11) for (−/−) and (+/+) neurons, respectively (fig. 6). These results indicate that GABA_A receptors present in (−/−) and (+/+) neurons are similarly influenced by pentobarbital.

Discussion

Our results show that GluR2 null mutant mice are more sensitive to the anesthetic effects of pentobarbital compared with wild-type littermates. In contrast, AMPA receptors in GluR2-deficient neurons were resistant to inhibition by barbiturates as indicated by a fourfold to sixfold increase in the IC₅₀ values for pentobarbital, thiopental, and phenobarbital. Taken together, these data demonstrate that the behavioral effects of barbiturates do not correlate with the inhibition of AMPA receptors in this mouse model. Although the rank order of potencies of the barbiturates for AMPA receptor inhibition (thiopental > pentobarbital > phenobarbital) is consistent with the behavioral potencies in animals and humans, this rank order also correlates with the modulation of GABA_A receptors, a primary target site for anesthetic drugs.

Previous behavioral studies suggest that inhibition of AMPA receptors contributes to the neurodepressive effects of barbiturates. The selective non-N-methyl-D-aspartate receptor antagonist, NBQX, administered intravenously in rats caused a dose-dependent increase in the duration of LORR caused by hexobarbital. Potentiation of the anesthetic effect of hexobarbital by NBQX, together with evidence that barbiturates inhibit the AMPA receptor in vitro, led to the suggestion that AMPA receptors contribute to the clinical properties of barbiturates. Alternatively, these behavioral data could also be interpreted as indicating that NBQX reduced the baseline level of excitatory neurotransmission, rendering the central nervous system more sensitive to the inhibitory GABAergic effects of barbiturates.

Electrophysiologic studies support a reduction in excitatory synaptic signaling in GluR2 null mutant mice as the amplitude of excitatory postsynaptic potentials and currents are reduced in hippocampal slices from these mice. This suggests that the absence of the GluR2 subunit is associated with a generalized reduction in excitatory neurotransmission, a state analogous to that induced by low concentrations of NBQX. Consequently, barbiturates may unmask an inherent susceptibility to neurodepressive drugs.

An alternative mechanism to account for the enhanced sensitivity to pentobarbital in GluR2 null mu-
tient mice is attributed to the modulation of polynu-
ronal networks. For example, in hippocampal circuits, inhibitory GABAergic interneurons are activated by glutamate. AMPA receptors present in these interneurons can contain GluR2 subunits as indicated by in situ hybridization and immunofluorescence labeling studies. In wild-type mice, pentobarbital blockade of AMPA receptors would reduce the activation of inhibitory interneurons, thereby reducing GABAergic transmission in these mice. In contrast, in GluR2 null mutant mice, barbiturate-resistant AMPA receptors would permit the persistent activation of inhibitory interneurons.

Thus, two mechanisms are postulated to account for our experimental results. The first attributes the enhanced sensitivity in GluR2 null mutant mice to a global reduction of excitatory neurotransmission and suggests that barbiturates act at non-AMPA receptors (such as GABA receptors). The second mechanism attributes the increased sensitivity in GluR2 null mutant mice to barbiturate-resistant AMPA receptors that activate inhibitory interneurons. Anesthetics that cause minimal inhibition of AMPA receptors and cause no differential modulation of wild-type and GluR2-deficient receptors could be used to distinguish between these two possibilities. We predict that if excitatory neurotransmission is globally reduced in GluR2 null mutant mice, then AMPA receptor-independent anesthetics would also be more po-
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Fig. 6. Concentration-response relationships for GABA and GABA plus pentobarbital-evoked currents recorded in acutely dissociated hippocampal pyramidal neurons. The peak current responses to GABA at 1, 3, 10, 30, 100, 300, 600, and 1,000 μM were normalized to the maximal response in the absence and presence of pentobarbital 30 μM.

A

Normalized Current

0 1 10 100 1000

Concentration of GABA (μM) +/- Pentobarbital

B

Normalized Current

0 0.2 0.4 0.6 0.8 1.0

Concentration of GABA (μM) +/- Pentobarbital

The presence of the GluR2 subunit was associated with a decrease in the number of drug binding sites or a change in the cooperativity or access to the binding site. Our results in native neurons from GluR2 mutant mice demonstrate a similar decrease in potency for barbiturate inhibition of GluR2-deficient receptors and a lower value for the Hill coefficient. However, deciphering the molecular mechanism(s) of inhibition by analyzing the concentration-response relation in mutant receptors is problematic because differences in agonist binding or gating of AMPA receptors may not be revealed by the shape of the concentration-response curve. Further studies are necessary to clarify the mechanisms of barbiturate inhibition of AMPA receptors.

In summary, our behavioral study indicates that GluR2 null mutant mice are more sensitive to barbiturates despite a reduced inhibition of GluR2-deficient AMPA receptors.
receptors by barbiturates. Our behavioral experiments were primarily designed to measure LORR as a surrogate end point for the loss of consciousness. However, the state of general anesthesia is comprised of multiple components, including hypnosis, amnesia, analgesia, and autonomic stability. Our results cannot be extrapolated to include the other components of general anesthesia such as analgesia or immobility in response to pain, because the absence or presence of the GluR2 subunit might differentially influence these end points. Nevertheless, from a clinical perspective, our results are compelling because a variety of neurologic insults, including ischemia and epilepsy, are associated with the down-regulation of the GluR2 subunit.38 Our data predict that disorders associated with a relative reduction in GluR2 subunit expression, including stroke,39,40 schizophrenia,11,12 Alzheimer’s disease,13 and amyotrophic lateral sclerosis,14,15 would be associated with a decreased dose requirement for anesthetic drugs.

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