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Barbiturates Require the N Terminus and First Transmembrane Domain of the δ Subunit for Enhancement of α1β3δ $GABA_A$ Receptor Currents*[S]

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$GABA_A$ receptors are composed predominantly of αβγ receptors, which mediate primarily synaptic inhibition, and αβδ receptors, which mediate primarily extrasynaptic inhibition. At saturating GABA concentrations, the barbiturate pentobarbital substantially increased the amplitude and desensitization of the α1β3δ receptor but not the α1β3γ2L receptor currents. To explore the structural domains of the δ subunit that are involved in pentobarbital potentiation and increased desensitization of α1β3δ currents, chimeric cDNAs were constructed by progressive replacement of γ2L subunit sequence with a δ subunit sequence or a δ subunit sequence with a γ2L subunit sequence, and HEK293T cells were co-transfected with α1 and β3 subunits or α1 and β3 subunits and a γ2L, δ, or chimeric subunit. Currents evoked by a saturating concentration of GABA or by co-application of GABA and pentobarbital were recorded using the patch clamp technique. By comparing the extent of enhancement and changes in kinetic properties produced by pentobarbital among chimeric and wild type receptors, we concluded that although potentiation of α1β3δ currents by pentobarbital required the δ subunit sequence from the N terminus to proline 241 in the first transmembrane domain (M1), increasing desensitization of α1β3δ currents required a δ subunit sequence from the N terminus to isoleucine 235 in M1. These findings suggest that the δ subunit N terminus and N-terminal portion of the M1 domain are, at least in part, involved in transduction of the allosteric effect of pentobarbital to enhance α1β3δ currents and that this effect involves a distinct but overlapping structural domain from that involved in altering desensitization.

γ-Aminobutyric acid, type A ($GABA_A$)[2] receptors, members of the Cys-loop receptor family, are heteropentameric ligand-gated chloride ion channels and play a critical role in mediating fast inhibition in the brain (1). Multiple $GABA_A$ receptor subunits as well as their subtypes have been identified (1, 2). Like the nicotinic cholinergic receptor, another member of the Cys-loop receptor family, each $GABA_A$ receptor subunit is thought to be composed of a large extracellular N terminus, four transmembrane domains (M1–M4), one extracellular M2–3 loop, two intracellular loops (M1–2 and M3–4), and an extracellular C terminus. Theoretically, an enormous number of receptors could be formed with different $GABA_A$ receptor subunit/subtype combinations. However, it has been proposed that αβγ and αβδ receptor isoforms are the predominant $GABA_A$ receptors in the brain (3). There is increasing evidence suggesting that αβγ $GABA_A$ receptors mainly locate within the synapses and mediate GABAergic phasic inhibition, whereas αβδ receptors are preferentially targeted to extra- or perisynaptic membranes and mediate tonic inhibition (4–6).

Barbiturates exert their effects in the brain by affecting $GABA_A$ receptor functions in a concentration-dependent manner. At lower concentrations, these compounds allosterically modulate $GABA_A$ receptors to potentiate GABAergic currents. At higher concentrations, they can directly activate $GABA_A$ receptors in the absence of GABA (2, 7, 8). It has been reported that the barbiturate pentobarbital substantially potentiated peak currents and increased desensitization of α1β3δ receptor currents evoked by saturating concentrations of GABA, but that these effects of pentobarbital were not observed with α1β3γ2L receptor currents at saturating GABA concentrations, although pentobarbital enhanced α1β3γ2L currents at subsaturating GABA concentrations (8), suggesting that the δ subunit rather than the γ2L subunit confers unique "modulatory potential" for this modulator at saturating GABA concentrations. The structural domains of the δ subunit that contribute to the potentiation and desensitization alterations by pentobarbital of currents evoked by saturating concentrations of GABA are currently unknown.

To explore this issue, we took advantage of the differential modulatory effects of pentobarbital on α1β3δ and α1β3γ2L receptors at a saturating GABA concentration to construct a series of chimeras by progressively replacing the rat γ2L subunit sequence with the corresponding rat δ subunit sequence or δ subunit sequence with the γ2L subunit sequence and co-transfected these chimeric subunits with wild type rat α1 and β3 subunits. Using an ultrafast drug delivery system and preapplication protocol, we performed whole cell patch clamp recordings of the currents evoked by a saturating concentration of GABA as well as by co-application of a saturating concentration of GABA and pentobarbital. By comparing the peak current amplitude enhancement and kinetic properties of chimeric

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[2] The abbreviations used are: $GABA_A$, γ-aminobutyric acid, type A; M, transmembrane domain; HEK293T, human embryonic kidney.

receptors with those of the wild type $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptors, we explored the structural basis for the differential effects of pentobarbital on $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptor currents evoked by saturating concentrations of GABA.

EXPERIMENTAL PROCEDURES

Expression of Wild Type and Chimeric Recombinant GABA$_A$ Receptors—Chimeric subunits were constructed by gradual replacement of the GABA$_A$ receptor γ2L subunit sequence with the corresponding δ subunit sequence or vice versa from the N terminus toward the C terminus using the splice overhang extension method. The cDNAs encoding rat wild type α1, β3, γ2L, and δ subunits as well as the chimeric subunits were cloned using the expression vector pCMVneo. The sequence of all of the wild type and chimeric subunits was verified by DNA sequencing (Vanderbilt DNA Sequencing Facility, Nashville, TN). Three δ-γ2L chimeras were constructed by progressively replacing the γ2L subunit sequence with the δ subunit sequence from the N toward the C terminus: δ-γ2L(M1e) (δG232-γY235), δ-γ2L(M1pre-iso) (δY234-γT237), and δ-γ2L(M1p) (δP241-γC244). Three reverse γ2L-δ chimeras were constructed by progressively replacing the δ subunit sequence with the γ2L subunit sequence from the N toward the C terminus: γ2L-δ(M1e′) (γG234-δV233), γ2L-δ(M1p′) (γP243-δS242), and γ2L-δ(M1i′) (γI257-δS256).

Human embryonic kidney (HEK293T) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 100 international units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) in an incubator at 37 °C with 5% CO_2 and 95% air. Cells were transfected with 2 μg of each cDNA encoding rat α1 and β3; α1, β3, and γ2L; α1, β3, and δ; or α1, β3, and chimeric GABA$_A$ receptor subunits using a modified calcium phosphate precipitation method (8). Two μg of pHOOK (Invitrogen) were co-transfected with the GABA$_A$ receptor subunits as a marker for the subsequent selection using an immunomagnetic bead separation method (9). Whole cell recordings were performed 24 h after the cells were selected.

Whole Cell Recordings—Whole cell currents were obtained using the patch clamp technique at room temperature. The external solution was composed of 142 mM NaCl, 1 mM $CaCl_2$, 6 mM $MgCl_2$, 8 mM KCl, 10 mM glucose, and 10 mM HEPES (pH 7.4, 323–329 mosmol). The recording electrodes were pulled from thin wall borosilicate glass tubes (World Precision Instruments, Sarasota, FL) on a P-87 Flaming Brown micropipette puller (Sutter Instruments, San Rafael, CA). The electrodes were fire-polished on an MF-9 microforge (Narishige, Tokyo, Japan). The resistances of the recording electrodes were 0.8–1.8 megohms after being filled with an internal solution consisting of 153 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM MgATP, and 5 mM EGTA (pH 7.3, 301–309 mosmol). Combination of the external and internal solutions produced a chloride equilibrium potential near 0 mV and a potassium equilibrium potential at −75 mV. Electrophysiological recordings were performed on either an Axopatch-1D or a 200A patch clamp amplifier (Molecular Devices, Union City, CA) and a Digidata 1200 series interface (Molecular Devices). Data were recorded on chart paper using a WR7400 arraycorder (Graphtec, Yokohama, Japan) as well as stored in a computer for offline analysis. All chemicals were purchased from Sigma-Aldrich. GABA and pentobarbital sodium salt were dissolved in water and prepared as stock solutions. Working solutions were made by diluting the stock solutions to desired concentrations with external solution on the day of the experiment. Drugs were applied by gravity via multibarrel tubes (two three-barrel square glass tubes glued together) connected to a Perfusion Fast-Step system (Warner Instruments, Hamden, CT). The 10–90% rise times of liquid junction currents were consistently <2 ms estimated by stepping a dilute external solution across an open electrode tip. GABA was applied for 4 s. The intervals between consecutive drug applications were at least 45 s to minimize desensitization accumulation.

Data Analysis—Whole cell currents were analyzed offline using Clampfit 8.1 (Molecular Devices). The peak currents were measured manually from the base line to the transient peak. The extent of enhancement of GABA currents by pentobarbital ($I_{PB}/I_{CONTROL}$) was calculated by dividing the peak current of co-application of GABA and pentobarbital by the peak current evoked by GABA alone. The extent of desensitization (% desensitization) was calculated by dividing the amount of current loss (peak current − current at the end of GABA/drug application) by peak current. Deactivation currents were fitted with one or two exponential components using the standard exponential Levenberg-Marquardt method in the form of $\Sigma a_n\tau_n$, where a denotes the relative amplitude of the exponential component, τ represents the time constant, and n is the number of exponential components. A weighted τ $(a_1{\cdot}\tau_1 + a_2{\cdot}\tau_2)/(a_1 + a_2)$ was used to compare the rate of deactivation, where a_1 and a_2 are the relative amplitudes of the exponential components at time 0.

Data were reported as mean ± S.E. A paired Student's t test was used to compare the changes prior to and after pentobarbital treatment. One-way analysis of variance followed by Newman-Keuls multiple comparison test was utilized to analyze the differences among wild type and chimeric receptors. The difference was considered to be statistically significant if $p < 0.05$.

RESULTS

Pentobarbital Modulated Wild Type and δ-γ2L Chimeric GABA$_A$ Receptor Current Amplitudes—The modulatory effects of pentobarbital were determined by preapplying pentobarbital (100 μM) for 1.5 s prior to jumping into a saturating concentration of GABA (1 mM) and pentobarbital (100 μM) (see Fig. 1 and Fig. 3). Pentobarbital did not potentiate $\alpha1\beta3\gamma2L$ receptor peak currents (Figs. 1*B* and 2*A*) but did substantially enhance those for $\alpha1\beta3\delta$ receptors (Figs. 1*F* and 2*A*), consistent with our previous report (8). Pentobarbital only slightly enhanced peak currents of $\alpha1\beta3$ receptors evoked by a saturating concentration of GABA. Although the enhancement by pentobarbital of $\alpha1\beta3$ receptor currents was not significantly different from that of $\alpha1\beta3\gamma2L$ receptor currents, it was, like $\alpha1\beta3\gamma2L$ receptors, significantly smaller than that of $\alpha1\beta3\delta$ receptors (supplemental Fig. 1), suggesting that the dramatic enhancement of $\alpha1\beta3\delta$ receptor currents by pentobarbital likely was conferred by incorporation of the δ subunit. Progressive replacement of the γ2L subunit N terminus and distal transmembrane M1

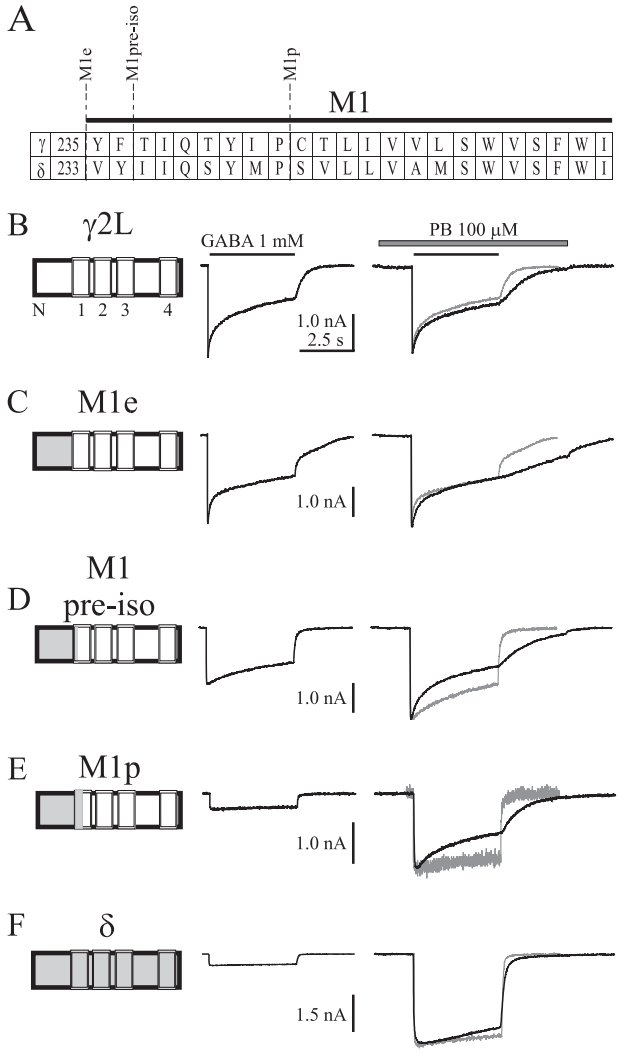

FIGURE 1. **Representative GABA current traces prior to and after pentobarbital modulation for wild type and δ-γ2L chimeric GABA$_A$ receptors.** *A*, amino acid alignment for M1 domain of γ2L and δ subunits. δ-γ2L chimera splice sites were indicated by *dashed lines*. *B–F*, representative whole cell current traces evoked by a saturating concentration of GABA (1 mM) (*left traces*) as well as co-application of GABA (1 mM) and pentobarbital (100 μM) with pentobarbital (*PB*, 100 μM) preapplied for 1.5 s (*right traces*). The GABA control currents (*gray traces*) were normalized to the currents evoked by co-application of GABA and pentobarbital to show the alterations of desensitization and deactivation. A *schematic* of GABA$_A$ receptor wild type subunit γ2L (*white shading*), δ (*gray shading*), or a chimeric subunit is depicted before each set of traces. *N* represents the N terminus of the subunit, and *numbers 1 to 4* denote M1–M4 of the subunit. The *solid line above* each current trace denotes the duration (4 s) of GABA application, and the *hatched bar* represents the duration of pentobarbital application. The horizontal time scale of *C*, *D*, *E*, and *F* is the same as that of *B*.

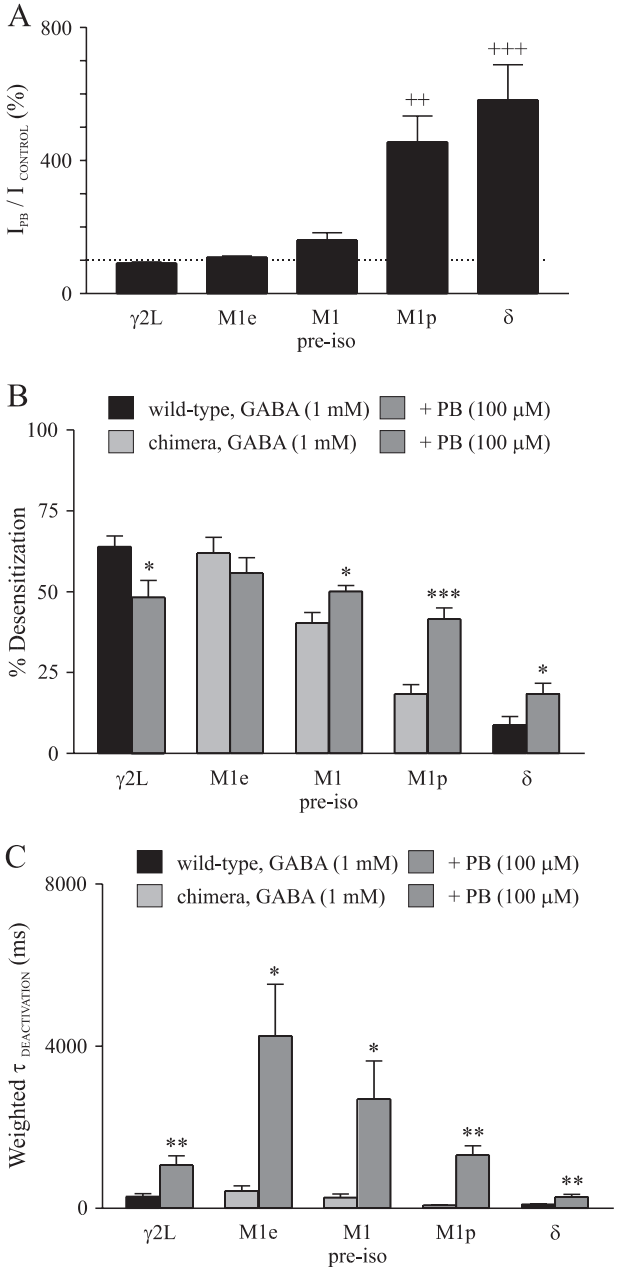

FIGURE 2. **Pentobarbital modulation of peak currents, desensitization, and deactivation of wild type and δ-γ2L chimeric receptors.** *A*, shown is the mean extent of enhancement by pentobarbital (*PB*) of wild type and δ-γ2L chimeric receptors. The *dashed line* indicates 100%. *B*, shown is a comparison of the mean desensitization prior to and after pentobarbital treatment among wild type and δ-γ2L chimeric receptors. *C*, the mean deactivation time constant was greater after pentobarbital treatment as compared with GABA control for wild type and δ-γ2L chimeric receptors. ++, significantly different from γ2L, M1e, or M1pre-iso at $p < 0.01$; +++, $p < 0.001$ (one-way analysis of variance followed by Newman-Keuls multiple comparison test); *, significantly different from GABA control at $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (paired Student's *t* test).

domain by the δ subunit sequence resulted in α1β3δ-γ2L chimeric receptors whose current enhancement was progressively increased by pentobarbital (Figs. 1, *C–E*, and 2*A*). Pentobarbital slightly potentiated the peak currents of α1β3δ-γ2L(M1e) receptors ($n = 5$), which contained the δ-γ2L(M1e) chimera with a δ subunit sequence in the entire extracellular N-terminal domain (Fig. 1*A*), but the extent of enhancement was not significantly different from α1β3γ2L receptors (Figs. 1*C* and 2*A*). For α1β3δ-γ2L(M1pre-iso) receptors ($n = 7$), which contained the δ-γ2L(M1pre-iso) chimera that advanced the δ subunit sequence just two amino acids into the M1 domain (Fig. 1*A*), the current enhancement by pentobarbital still was not significantly different from that of α1β3γ2L receptors (Figs. 1*D* and 2*A*). There was no significant difference in the current enhancements between α1β3δ-γ2L(M1pre-iso) and α1β3δ-γ2L(M1e) receptors (Fig. 2*A*). The current enhancement induced by pentobarbital for α1β3δ-γ2L(M1p) receptors (454.8 ± 78.6%, $n = 7$), which contained the δ-γ2L(M1p) chi-

mera that advanced the δ subunit sequence seven more amino acids into the M1 domain (Fig. 1*A*), was greater than that of α1β3γ2L, α1β3δ-γ2L(M1e), or α1β3δ-γ2L(M1pre-iso) receptors ($p < 0.01$), and this enhancement was not significantly different from that of wild type α1β3δ receptors (582.6 ± 105.1%, $n = 8$) (Figs. 1, *E* and *F*, and 2*A*). Note that α1β3 currents evoked by a saturating concentration of GABA and pentobarbital exhibited multiphasic desensitization (supplemental Fig. 1, *right trace*). This was not observed with wild type α1β3γ2L and α1β3δ receptor as well as chimeric receptor currents (Figs. 1 and 3, *right traces*), indicating that the γ2L, δ, or chimeric subunit assembled with α1 and β3 subunits to form ternary receptors with negligible binary α1β3 receptors.

Pentobarbital Modulated the Desensitization and Deactivation of Wild Type and δ-γ2L Chimeric GABA$_A$ Receptors—As reported previously (10), wild type and chimeric GABA$_A$ receptor currents evoked by a saturating concentration of GABA exhibited different extents of desensitization (Fig. 1, *B–F, left traces*). Mean % desensitization of α1β3γ2L or α1β3δ-γ2L(M1e) currents was greater than that of α1β3δ-γ2L(M1pre-iso), α1β3δ-γ2L(M1p), or α1β3δ currents (Fig. 2*B*; $p < 0.001$). Desensitization of α1β3δ-γ2L(M1pre-iso) currents was greater than that of α1β3δ-γ2L(M1p) or α1β3δ currents ($p < 0.001$), and desensitization of α1β3δ-γ2L(M1p) currents was greater than that of α1β3δ currents ($p < 0.05$) (Fig. 2*B*). Desensitization was not significantly different between α1β3γ2L and α1β3δ-γ2L(M1e) currents.

Consistent with our previous report (8), pentobarbital significantly decreased the desensitization of α1β3γ2L currents but increased that of α1β3δ currents evoked by a saturating concentration of GABA (Figs. 1, *B* and *F*, and 2*B*). Desensitization of α1β3δ-γ2L(M1e) currents was not significantly altered by pentobarbital (Figs. 1*C* and 2*B*). However, pentobarbital increased the desensitization of α1β3δ-γ2L(M1pre-iso) currents (40.4 ± 3.3% *versus* 50.1 ± 1.8%) ($p < 0.05$) (Figs. 1*D* and 2*B*). Desensitization of α1β3δ-γ2L(M1p) currents was increased by pentobarbital from 18.4 ± 2.9% to 41.5 ± 3.6% ($p < 0.001$) (Figs. 1*E* and 2*B*).

Currents evoked by a saturating concentration of GABA also deactivated differently among wild type and δ-γ2L chimeric receptors (Fig. 1, *B--F, left traces*). The mean deactivation time constant of α1β3δ-γ2L(M1e) currents (438.9 ± 118.3 ms) was greater than that of α1β3δ-γ2L(M1p) (77.6 ± 9.0 ms) or α1β3δ (95.7 ± 12.6 ms) currents ($p < 0.01$), although it was not significantly different from that of α1β3γ2L (292.9 ± 69.4 ms) or α1β3δ-γ2L(M1pre-iso) (269.7 ± 86.0 ms) currents. The deactivation time constant was not significantly different among α1β3γ2L, α1β3δ-γ2L(M1pre-iso), α1β3δ-γ2L(M1p), and α1β3δ currents (Fig. 2*C*).

Pentobarbital significantly prolonged the deactivation of all of the wild type and δ-γ2L chimeric receptor currents (Fig. 2*C*). The deactivation time constant of α1β3γ2L currents was increased by pentobarbital to 1072.5 ± 220.9 ms ($p < 0.01$) (Figs. 1*B* and 2*C*). Pentobarbital increased the deactivation time constant of α1β3δ-γ2L(M1e) currents to 4253.9 ± 1274.9 ms ($p < 0.05$) (Figs. 1*C* and 2*C*). An increase in deactivation time constant also was observed for α1β3δ-γ2L(M1pre-iso) (2698.6 ± 935.0 ms) ($p < 0.05$), α1β3δ-γ2L(M1p) (1315.0 ± 225.0 ms) ($p < 0.01$), and α1β3δ (286.3 ± 62.1 ms) ($p < 0.01$) currents (Figs. 1, *D–F*, and 2*C*).

Pentobarbital Modulated the Current Amplitudes of γ2L-δ Chimeric GABA$_A$ Receptors—The results obtained using δ-γ2L chimeras suggested that the N terminus as well as the N-terminal portions of the M1 domain of the δ subunit were critical for pentobarbital modulation. However, all of the constructs contained the δ subunit sequence in the N terminus, so it was not possible to isolate the role of the M1 domain. Is this domain sufficient to support δ subunit-like modulation by pentobarbital? One way to address this issue is to examine reverse γ2L-δ chimeras.

The M1e′ γ2L-δ chimera contained the γ2L subunit sequence in the entire extracellular N-terminal domain (Fig. 3*A*). Pentobarbital slightly enhanced peak currents of the α1β3γ2L-δ(M1e′) receptors (130.4 ± 14.1%, $n = 6$), but this enhancement was substantially smaller than that of α1β3δ receptors ($p < 0.001$) (Figs. 3, *B* and *C*, and 4*A*). The M1p′ chimera advanced the γ2L subunit sequence nine amino acids into the M1 domain (Fig. 3*A*). The α1β3γ2L-δ(M1p′) currents ($n = 8$) were slightly enhanced by pentobarbital, whose enhancement was much smaller than that of α1β3δ receptors ($p < 0.001$) (Figs. 3, *B* and *D*, and 4*A*). The M1i′ chimera advanced the γ2L subunit sequence fourteen more amino acids into the M1 domain (Fig. 3*A*), and pentobarbital minimally enhanced α1β3γ2L-δ(M1i′) currents ($n = 7$) (Figs. 3*E* and 4*A*). The current enhancement evoked by pentobarbital of α1β3γ2L-δ(M1e′), α1β3γ2L-δ(M1p′), or α1β3γ2L-δ(M1i′) receptors was not significantly different from that of α1β3γ2L receptors (Fig. 4*A*).

Pentobarbital Modulated the Desensitization and Deactivation of γ2L-δ Chimeric GABA$_A$ Receptors—The currents of α1β3γ2L-δ(M1e′), α1β3γ2L-δ(M1p′), and α1β3γ2L-δ(M1i′) receptors evoked by 1 mM GABA exhibited substantial desensitization, which was quite different from that of α1β3δ receptors (Fig. 3, *B–E, left traces*). Instead, the desensitization of these receptor currents was similar to that of wild type α1β3γ2L receptors (Fig. 3, *C--F, left traces*), and the mean desensitization of these receptors was not significantly different from one another (Fig. 4*B*). Pentobarbital decreased the desensitization of both α1β3γ2L-δ(M1e′) (66.0 ± 3.9% *versus* 51.7 ± 8.3%) ($p < 0.05$) and α1β3γ2L-δ(M1p′) (70.7 ± 1.9% *versus* 48.8 ± 5.6%) ($p < 0.01$) currents, as was observed for α1β3γ2L currents (Fig. 4*B*). However, the desensitization of α1β3γ2L-δ(M1i′) currents was not altered significantly by pentobarbital (Fig. 4*B*).

Deactivation of α1β3γ2L-δ(M1e′), α1β3γ2L-δ(M1p′), or α1β3γ2L-δ(M1i′) currents was similar (Fig. 3, *C–E, left traces*), and the mean deactivation time constants of these receptors were not significantly different from either α1β3δ or α1β3γ2L receptors (Fig. 4*C*). Pentobarbital significantly prolonged deactivation of the α1β3γ2L-δ(M1e′), α1β3γ2L-δ(M1p′), and α1β3γ2L-δ(M1i′) currents (Fig. 4*C*). The deactivation time constant of α1β3γ2L-δ(M1e′) currents was increased from 160.5 ± 32.2 ms to 1932.6 ± 534.7 ms ($p < 0.05$) by pentobarbital (Figs. 3*C* and 4*C*). Pentobarbital increased the deactivation time constant of α1β3γ2L-δ(M1p′) currents from 171.8 ± 25.5 ms to 1600.8 ± 203.5 ms ($p < 0.001$) (Figs. 3*D*

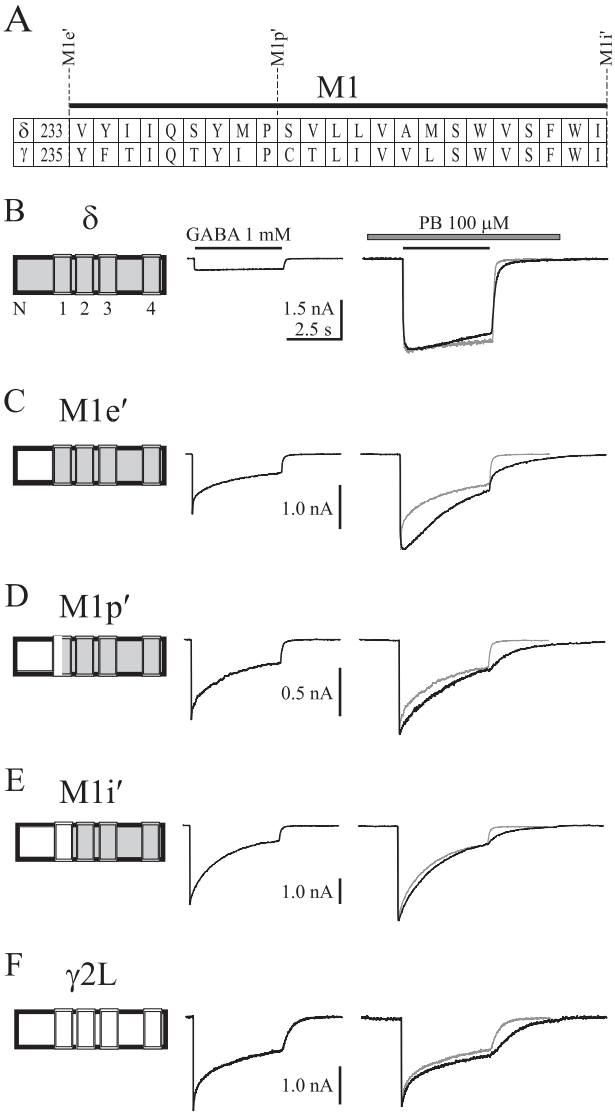

FIGURE 3. **Representative GABA current traces prior to and after pentobarbital modulation for wild type and γ2L-δ chimeric receptors.** *A*, amino acid alignment for M1 domain of δ and γ2L subunits. γ2L-δ chimera splice sites were indicated by *dashed lines*. *B–F*, representative whole cell current traces evoked by a saturating concentration of GABA (1 mM) (*left traces*) as well as co-application of GABA (1 mM) and pentobarbital (*PB*, 100 μM) with pentobarbital (100 μM) preapplied for 1.5 s (*right traces*). The GABA control currents (*gray traces*) were normalized to the currents evoked by co-application of GABA and pentobarbital to show the alterations of desensitization and deactivation. A *schematic* of the GABA$_A$ receptor wild type subunit δ (*gray shading*), γ2L (*white shading*), or chimeric subunit was shown before each set of traces. *N* represents N terminus of the subunit, and *numbers 1–4* denote M1–M4 of the subunit. The *solid line above each current trace* denotes the duration (4 s) of GABA application, and the *hatched bar* represents the duration of pentobarbital application. The horizontal time scale of *C*, *D*, *E*, and *F* is the same as that of *B*.

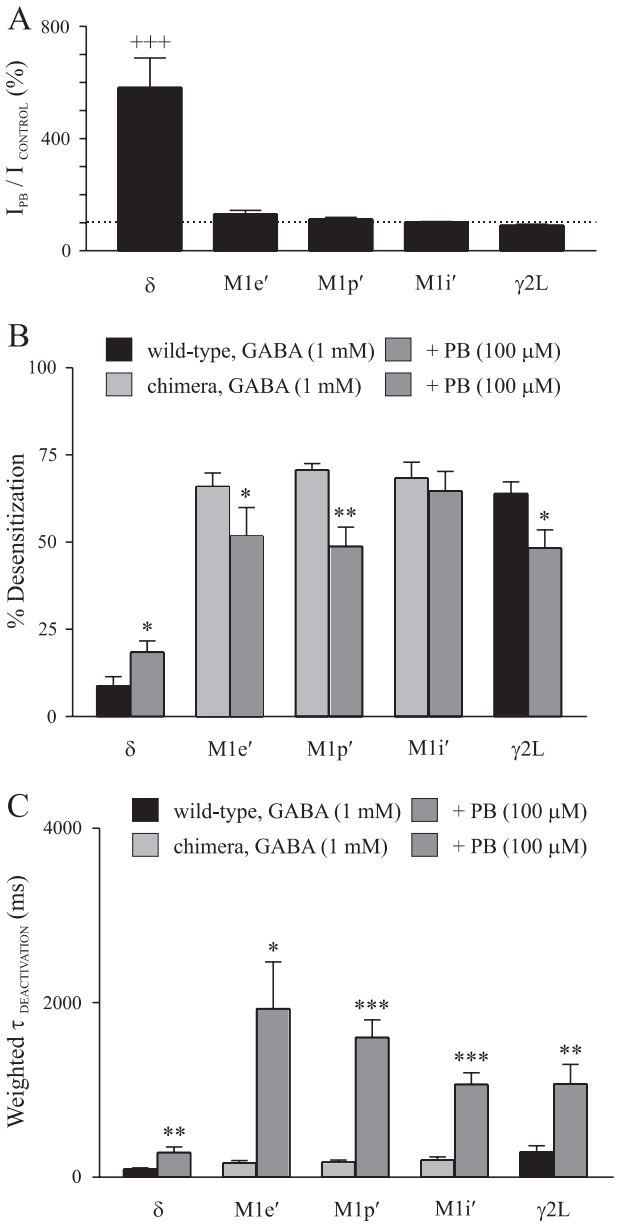

FIGURE 4. **Pentobarbital modulation of peak currents, desensitization and deactivation of wild type and γ2L-δ chimeric receptors.** *A*, shown is the mean extent of enhancement by pentobarbital (*PB*) of wild type and γ2L-δ chimeric receptors. The *dashed line* indicates 100%. *B*, shown is the comparison of the mean desensitization prior to and after pentobarbital treatment among wild type and γ2L-δ chimeric receptors. *C*, the mean deactivation time constant was greater after pentobarbital treatment as compared with GABA control for wild type and γ2L-δ chimeric receptors. +++, significantly different from M1e′, M1p′, M1i′, and γ2L at $p < 0.001$ (one-way analysis of variance followed by Newman-Keuls multiple comparison test); *, significantly different from GABA control at $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (paired Student's *t* test).

and 4*C*). The deactivation time constant of α1β3γ2L-δ(M1i′) currents in the presence of pentobarbital was increased to 1065.4 ± 134.3 ms from GABA control of 199.8 ± 35.0 ms ($p < 0.001$) (Figs. 3*E* and 4*C*).

DISCUSSION

Pentobarbital Required the N Terminus and N-terminal Portion of the δ Subunit M1 Domain from Valine 233 to Proline 241 for Potentiation of α1β3δ Receptor Currents—A large body of literature demonstrates that αβδ receptors are selectively enhanced by a variety of structurally different compounds as compared with αβγ receptors (8, 11–20). Currents evoked by a saturating concentration of GABA were consistently smaller for α1β3δ receptors than for α1β3γ2L receptors (8, 15, 19, 21). Single channel recordings found that αβδ currents exhibited brief openings, whereas αβγ currents displayed bursting openings with longer mean open duration (8, 15, 18, 22–24). In addition, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol and pentobarbital evoked greater currents than GABA from αβδ

receptors (8, 12, 18). These studies strongly suggest that GABA is a partial agonist for $\alpha\beta\delta$ receptors such that it leaves substantial "modulatory capacity" for many allosteric modulators (16).

Several modulators, including pentobarbital, enhanced $\alpha\beta\delta$ currents evoked by a saturating concentration of GABA mainly by increasing channel gating efficacy (8, 15, 18, 20). The present study sought to determine the structural domains of the δ subunit involved in transduction of the allosteric effect of pentobarbital to current enhancement and alteration of desensitization. We observed that the enhancement by pentobarbital of currents evoked by a saturating concentration of GABA for $\alpha1\beta3\delta$-$\gamma2$L(M1p) receptors was not different from that for $\alpha1\beta3\delta$ receptors, suggesting that the δ subunit domain from the beginning of the N terminus to proline 241 in M1 is sufficient for maximal potentiation by pentobarbital. The enhancement of the reversed chimeric $\alpha1\beta3\gamma2$L-δ(M1p′) receptor currents by pentobarbital was not different from $\alpha1\beta3\gamma2$L receptors, supporting the idea that the δ subunit domain from the beginning of N terminus to the proline 241 in M1 is necessary and sufficient for maximal potentiation by pentobarbital. The enhancement by pentobarbital of $\alpha1\beta3\gamma2$L-δ(M1e′) currents was <25% of $\alpha1\beta3\delta$ receptors, implying that the δ subunit N terminus plays a critical role in pentobarbital enhancement. However, $\alpha1\beta3\delta$-$\gamma2$L(M1e) currents were only slightly potentiated by pentobarbital. These observations lead to the conclusion that both the N terminus and M1 domain from valine 233 to proline 241 of the δ subunit are necessary and sufficient for pentobarbital potentiation of $\alpha1\beta3\delta$ currents evoked by saturating concentrations of GABA.

Both GABA$_A$ and nicotinic cholinergic receptors are members of the Cys-loop receptor family and are proposed to share similar topological structures. It was reported that some of the residues in the N-terminal portion of M1 domain of the nicotinic cholinergic receptor were exposed in the channel lining and might be involved in gating (25). An invariant proline residue in M1 domain of nicotinic cholinergic receptor subunits is critically coupled with ligand binding and channel gating (26). Mutation of the M1 proline (equivalent to proline 241 in the δ subunit) in $\alpha1$ or $\beta1$ subunits reduced the enhancement by barbiturates of submaximal GABA-evoked currents in $\alpha1\beta1\gamma2$ receptors (27), suggesting that the M1 proline is involved in transduction of allosteric effect of barbiturates. An invariant glycine residue (equivalent to glycine 232 in the δ subunit) at the entrance to M1 of GABA$_A$ receptors also might be involved in transduction of allosteric effect of anesthetics including pentobarbital (28). The present finding that the M1 domain from valine 233 to proline 241 of the δ subunit contributes to the pentobarbital potentiation of $\alpha1\beta3\delta$ currents implies that some of these residues may be directly involved in the transduction of the pentobarbital allosteric effect to channel gating. Furthermore, in addition to the M1 domain residues, we also demonstrated that the N terminus of the δ subunit contributed to the pentobarbital potentiation of $\alpha1\beta3\delta$ currents. Multiple residues in the GABA$_A$ receptor N terminus were reported to couple with the M2–3 linker to affect channel gating (29). The finding that the structural domains of the δ subunit beyond M1 may not be critically involved in transduction of the pentobarbital allosteric effect suggests that the δ subunit N terminus may be able to interact with either the δ or $\gamma2$L subunit M2–3 linker. We cannot, however, rule out an interaction between these two domains that occurs via residues that are conserved between the δ and $\gamma2$L subunits.

Like the nicotinic cholinergic receptor (30–34), the M2 domains form the ion conduction pathway and the channel gate of GABA$_A$ receptors. Pentobarbital at modulating concentrations did not seem to interfere with the gate via this domain because the M2 domain of δ subunit was not required for potentiation of $\alpha1\beta3\delta$ currents evoked by a saturating concentration of GABA. Some residues in transmembrane regions (including M2 domain) of α and β subunits have been reported to be involved in anesthetic modulation (35–39). These studies suggested that allosteric modulation of GABA$_A$ receptors by anesthetics is subunit-dependent and that different anesthetics may have different actions on GABA$_A$ receptors.

It is possible that the domain from the beginning of the N terminus to M1 proline 241 might only confer $\alpha1\beta3\delta$ receptors with a "partial agonist" property, such that these residues were not necessarily involved in transduction of pentobarbital allosteric effects. If this was the case, the similar structural domain is likely to be involved in the enhancement of $\alpha1\beta3\delta$ receptor currents by different modulators. However, we found that the neurosteroid tetrahydrodeoxycorticosterone required structural domains beyond M2 for full potentiation of $\alpha1\beta3\delta$ currents induced by a saturating concentration of GABA.[3] In addition, mutation of the M1 proline in $\alpha1$ or $\beta1$ of the GABA$_A$ receptor only reduced the enhancement by barbiturates without interfering with that by neurosteroids (27). Therefore, this required domain from the beginning of the N terminus to M1 proline 241 is to some extent "specific" for pentobarbital modulation. These residues are unlikely to contribute to the formation of a barbiturate binding site as previous studies in δ subunit knock-out mice suggest that general anesthetics like barbiturates, etomidate, and propofol are not selective for $\alpha\beta\delta$ receptors, although there may be some δ subunit selectivity for neurosteroids (40, 41).

Interestingly, it has been reported recently that general anesthetics like propofol, etomidate, and tetrahydrodeoxycorticosterone enhanced both $\alpha4\beta3$ and $\alpha4\beta3\delta$ receptors to the same extent when applied with saturating concentrations of GABA (42), indicating that it is the $\alpha4$ instead of the δ subunit that confers current enhancement by general anesthetics. However, in the current study, we demonstrated that δ subunits play an important role in pentobarbital potentiation of $\alpha1\beta3\delta$ currents, as pentobarbital greatly enhanced $\alpha1\beta3\delta$ currents but only slightly enhanced $\alpha1\beta3$ currents. Therefore, α subunits may play a role in modulation of $\alpha\beta\delta$ receptors by anesthetics.

Pentobarbital Required the N Terminus and N-terminal Portion of the δ Subunit M1 Domain from Valine 233 to Isoleucine 235 to Alter $\alpha1\beta3\delta$ Current Desensitization—Pentobarbital decreased the desensitization of $\alpha1\beta3\gamma2$L currents but

[3] M. T. Bianchi and R. L. Macdonald, unpublished observations.

increased that of $\alpha1\beta3\delta$ currents induced by a saturating concentration of GABA (8, this study). Pentobarbital did not alter desensitization of $\alpha1\beta3\delta$-$\gamma2$L(M1e) receptor currents and decreased desensitization of most $\alpha1\beta3\gamma2$L-δ chimeric receptor currents, suggesting that the N terminus of the δ subunit may be involved in the pentobarbital-induced increase of desensitization. Some residues in the δ subunit M1 domain may also cause the desensitization increase since an increase in desensitization by pentobarbital was observed for $\alpha1\beta3\delta$-$\gamma2$L(M1pre-iso) and $\alpha1\beta3\delta$-$\gamma2$L(M1p) receptor currents. These data imply that δ subunit residues from the beginning of the N terminus to M1 isoleucine 235 are a requirement for pentobarbital to increase $\alpha1\beta3\delta$ current desensitization. Our previous study (10) and the current study (Fig. 1*D*, *left trace*) showed that these δ subunit residues were required to abolish the fast desensitization of currents evoked by a saturating concentration of GABA. One possibility is that pentobarbital might modulate the rate constants into and out of the desensitized states to increase the occupancy of the desensitized states. It was reported that the desensitized state of nicotinic cholinergic receptors was conferred by some residues lining the inside of the ion conduction pathway and that the desensitized state was structurally different from other states (43). We demonstrated here that the structural domain of the δ subunit involved in increasing desensitization overlapped with but was distinct from that involved in transduction of the allosteric effect of pentobarbital.

Pentobarbital Prolonged Deactivation of All Wild Type and Chimeric Receptor Currents—Pentobarbital was reported to prolong the deactivation of $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2$L receptor currents evoked by a saturating concentration of GABA (8). It was proposed that prolongation of deactivation is "coupled" with increased desensitization (23, 44). Consistent with this idea, pentobarbital prolonged deactivation and enhanced desensitization of $\alpha1\beta3\delta$ receptors as well as most of the δ-$\gamma2$L chimeric receptors. However, "uncoupling" of desensitization and deactivation was also observed. Pentobarbital decreased the desensitization of $\alpha1\beta3\gamma2$L receptors as well as most of the $\alpha1\beta3\gamma2$L-δ chimeric receptors and did not modify the desensitization of $\alpha1\beta3\delta$-$\gamma2$L(M1e) receptors, although deactivation of these receptors was prolonged by pentobarbital. These data further support the idea that other factors in addition to desensitization affect deactivation. Deactivation has been demonstrated to be prolonged by increased gating efficacy and agonist affinity (45). Interestingly, we observed that pentobarbital dramatically prolonged deactivation of $\alpha1\beta3\delta$-$\gamma2$L(M1e) receptor currents, but that peak currents and current desensitization were not modulated dramatically by pentobarbital. One possible explanation may be that pentobarbital slowed GABA unbinding, which has been reported for another general anesthetic drug, halothane (46).

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REFERENCES

1. Olsen, R. W., and Macdonald, R. L. (2002) *Glutamate and GABA Receptors and Transporters: Structure, Function, and Pharmacology*, pp. 202–235, Taylor & Francis, London
2. Hevers, W., and Lüddens, H. (1998) *Mol. Neurobiol.* **18,** 35–86
3. McKernan, R. M., and Whiting, P. J. (1996) *Trends Neurosci.* **19,** 139–143
4. Mody, I., and Pearce, R. A. (2004) *Trends Neurosci.* **27,** 569–575
5. Farrant, M., and Nusser, Z. (2005) *Nat. Rev. Neurosci.* **6,** 215–229
6. Belelli, D., Harrison, N. L., Maguire, J., Macdonald, R. L., Walker, M. C., and Cope, D. W. (2009) *J. Neurosci.* **29,** 12757–12763
7. Schulz, D. W., and Macdonald, R. L. (1981) *Brain Res.* **209,** 177–188
8. Feng, H. J., Bianchi, M. T., and Macdonald, R. L. (2004) *Mol. Pharmacol.* **66,** 988–1003
9. Greenfield, L. J., Jr., Sun, F., Neelands, T. R., Burgard, E. C., Donnelly, J. L., and MacDonald, R. L. (1997) *Neuropharmacology* **36,** 63–73
10. Bianchi, M. T., Haas, K. F., and Macdonald, R. L. (2001) *J. Neurosci.* **21,** 1127–1136
11. Lees, G., and Edwards, M. D. (1998) *Anesthesiology* **88,** 206–217
12. Adkins, C. E., Pillai, G. V., Kerby, J., Bonnert, T. P., Haldon, C., McKernan, R. M., Gonzalez, J. E., Oades, K., Whiting, P. J., and Simpson, P. B. (2001) *J. Biol. Chem.* **276,** 38934–38939
13. Sundstrom-Poromaa, I., Smith, D. H., Gong, Q. H., Sabado, T. N., Li, X., Light, A., Wiedmann, M., Williams, K., and Smith, S. S. (2002) *Nat. Neurosci.* **5,** 721–722
14. Thompson, S. A., Wingrove, P. B., Connelly, L., Whiting, P. J., and Wafford, K. A. (2002) *Mol. Pharmacol.* **61,** 861–869
15. Wohlfarth, K. M., Bianchi, M. T., and Macdonald, R. L. (2002) *J. Neurosci.* **22,** 1541–1549
16. Bianchi, M. T., and Macdonald, R. L. (2003) *J. Neurosci.* **23,** 10934–10943
17. Wallner, M., Hanchar, H. J., and Olsen, R. W. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 15218–15223
18. Akk, G., Bracamontes, J., and Steinbach, J. H. (2004) *J. Physiol.* **556,** 387–399
19. Feng, H. J., and Macdonald, R. L. (2004) *Mol. Pharmacol.* **66,** 1517–1524
20. Feng, H. J., and Macdonald, R. L. (2004) *J. Neurophysiol.* **92,** 1577–1585
21. Saxena, N. C., and Macdonald, R. L. (1994) *J. Neurosci.* **14,** 7077–7086
22. Fisher, J. L., and Macdonald, R. L. (1997) *J. Physiol.* **505,** 283–297
23. Haas, K. F., and Macdonald, R. L. (1999) *J. Physiol.* **514,** 27–45
24. Steinbach, J. H., and Akk, G. (2001) *J. Physiol.* **537,** 715–733
25. Akabas, M. H., and Karlin, A. (1995) *Biochemistry* **34,** 12496–12500
26. England, P. M., Zhang, Y., Dougherty, D. A., and Lester, H. A. (1999) *Cell* **96,** 89–98
27. Greenfield, L. J., Jr., Zaman, S. H., Sutherland, M. L., Lummis, S. C., Niemeyer, M. I., Barnard, E. A., and Macdonald, R. L. (2002) *Neuropharmacology* **42,** 502–521
28. Carlson, B. X., Engblom, A. C., Kristiansen, U., Schousboe, A., and Olsen, R. W. (2000) *Mol. Pharmacol.* **57,** 474–484
29. Kash, T. L., Jenkins, A., Kelley, J. C., Trudell, J. R., and Harrison, N. L. (2003) *Nature* **421,** 272–275
30. Filatov, G. N., and White, M. M. (1995) *Mol. Pharmacol.* **48,** 379–384
31. Labarca, C., Nowak, M. W., Zhang, H., Tang, L., Deshpande, P., and Lester, H. A. (1995) *Nature* **376,** 514–516
32. Wilson, G. G., and Karlin, A. (1998) *Neuron* **20,** 1269–1281
33. Zhang, H., and Karlin, A. (1998) *Biochemistry* **37,** 7952–7964
34. Miyazawa, A., Fujiyoshi, Y., and Unwin, N. (2003) *Nature* **423,** 949–955
35. Nishikawa, K., and Harrison, N. L. (2003) *Anesthesiology* **99,** 678–684
36. Li, G. D., Chiara, D. C., Sawyer, G. W., Husain, S. S., Olsen, R. W., and Cohen, J. B. (2006) *J. Neurosci.* **26,** 11599–11605
37. Campagna-Slater, V., and Weaver, D. F. (2007) *Neurosci. Lett.* **418,** 28–33
38. Richardson, J. E., Garcia, P. S., O'Toole, K. K., Derry, J. M., Bell, S. V., and Jenkins, A. (2007) *Anesthesiology* **107,** 412–418
39. Stewart, D., Desai, R., Cheng, Q., Liu, A., and Forman, S. A. (2008) *Mol.*

Pharmacol. **74,** 1687–1695
40. Mihalek, R. M., Banerjee, P. K., Korpi, E. R., Quinlan, J. J., Firestone, L. L., Mi, Z. P., Lagenaur, C., Tretter, V., Sieghart, W., Anagnostaras, S. G., Sage, J. R., Fanselow, M. S., Guidotti, A., Spigelman, I., Li, Z., DeLorey, T. M., Olsen, R. W., and Homanics, G. E. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 12905–12910
41. Stell, B. M., Brickley, S. G., Tang, C. Y., Farrant, M., and Mody, I. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 14439–14444
42. Meera, P., Olsen, R. W., Otis, T. S., and Wallner, M. (2009) *Neuropharmacology* **56,** 155–160
43. Wilson, G., and Karlin, A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 1241–1248
44. Jones, M. V., and Westbrook, G. L. (1995) *Neuron* **15,** 181–191
45. Bianchi, M. T., Botzolakis, E. J., Haas, K. F., Fisher, J. L., and Macdonald, R. L. (2007) *J. Physiol.* **584,** 769–787
46. Li, X., and Pearce, R. A. (2000) *J. Neurosci.* **20,** 899–907