Pentobarbital Decreases the $\gamma$-Aminobutyric Acid$\alpha$ Receptor Subunit $\gamma$-2 Long/Short mRNA Ratio by a Mechanism Distinct from Receptor Occupation$^1$


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ABSTRACT

Treatment with pentobarbital of primary cultured cerebellar granule cells decreased the $\gamma$-aminobutyric acid, (GABA$\alpha$) receptor subunit $\gamma$-2 long/short ($\gamma$-2L/S) mRNA ratio. A high dose of pentobarbital (500 $\mu$M) decreased the $\gamma$-2L/S ratio by 64%; the decrease was dose and time dependent and reversible. ($\gamma$)-Hexobarbital (500 $\mu$M), the less potent stereoisomer for GABA$\alpha$ receptor activation, decreased the ratio slightly (30%) but significantly more than ($\gamma$)-hexobarbital (20%). Other GABA$\alpha$ receptor activators had no (100 mM ethanol) or little (2 $\mu$M $\alpha$-pregnane-3$\alpha$-ol-20-one) effect on the $\gamma$-2L/S ratio. Furthermore, picrotoxin (10 $\mu$M), which blocks the GABA- and pentobarbital-activated GABA$\alpha$ receptor channel, neither changed the $\gamma$-2L/S ratio nor blocked the pentobarbital-induced changes. These data suggest that barbiturates alter the $\gamma$-2L/S mRNA ratio by a mechanism that does not require GABA$\alpha$ receptor activation. The $\gamma$-2L/S subunit mRNA includes an exon encoding an octapeptide that contains a protein kinase C phosphorylation consensus site. This exon-encoded peptide, occurring in the putative intracellular loop, can be phosphorylated, and in vitro, this phosphorylation has been shown to have functional consequences. This is the first report of a drug-induced alteration in receptor mRNA splicing. Furthermore, the changes in the $\gamma$-2L/S ratio produced by pentobarbital exposure may have significant effects on the function of an important brain protein, the GABA$\alpha$ receptor.

The family of GABA$\alpha$ receptors are ligand-gated chloride channels formed from combinations of five of the $>$13 mammalian receptor subunits (alpha 1–6, beta 1–3, gamma 1–3, delta and rho) in pentameric arrays (Olsen and Tobin, 1990). This multiplicity of subunits provides one basis for the diverse pharmacology of the GABA$\alpha$ receptors (Tyndale et al., 1995b). Further diversity can result from splicing variants of individual receptor subunit mRNAs and intracellular phosphorylation of the GABA$\alpha$ receptor subunits (Lin et al., 1996; Moss et al., 1992b; Swope et al., 1992).

We tested the chronic effects of two GABA$\alpha$ receptor agonists on receptor adaptation in primary cultures of cerebellar granule cells. Initially, changes in mRNA for 13 subunits were assessed after chronic treatment. The only major change observed, however, was in the ratio of the long and short forms of mRNA for the $\gamma$-2 subunit. The $\gamma$-2 subunit mRNA exists in two forms, a long form ($\gamma$-2L), whose mRNA contains a 24-nt exon, and a short form ($\gamma$-2S), which does not contain this exon (Whiting et al., 1990; Kofuji et al., 1991). The additional exon of $\gamma$-2L encodes an octapeptide that lies within the putative cytoplasmic domain, between the third and fourth transmembrane regions (Whiting et al., 1990; Kofuji et al., 1991). The octapeptide includes a consensus phosphorylation site for PKC, with a serine in position 343. Inclusion or exclusion of this octapeptide and its phosphorylation site may alter the phosphorylation of the resulting receptor and lead to altered receptor function (Kellenberger et al., 1992; Leidenheimer et al., 1992; Lin et al., 1996; Machu et al., 1993; Moss et al., 1992a, 1992b). Compounds that alter the ratio of $\gamma$-2L/S mRNAs may also alter the functional properties of GABA$\alpha$ receptors; therefore, such compounds will enable further exploration of the role of receptor phosphorylation in receptor function.

In the current study, we used RT-PCR to investigate the

ABBREVIATIONS: RT-PCR, reverse transcription-polymerase chain reaction; GABA, $\gamma$-aminobutyric acid; PB, pentobarbital; PX, picrotoxin; HX, hexobarbital; NNE, non-neuronal enolase; PKC, protein kinase C; L/S, long/short; nt, nucleotide(s).
changes in the gamma-2L/S mRNA ratio after chronic exposure of cerebellar granule cells to various agents that act at GABA_A receptors. Previous biochemical, molecular, pharmacological and electrophysiological studies have demonstrated that cerebellar granule cells are a good model system for the study of GABA_A receptors (e.g., Beattie and Siegel, 1993; Bovlin et al., 1992; Iorio et al., 1992, 1993; Kaneda et al., 1995). Some of these results have been previously reported in abstract form (Tyndale et al., 1985a, 1996).

Methods

Materials. Basal essential medium and fetal bovine serum were obtained from GIBCO BRL (Grand Island, NY). Allopregnanolone (3a-hydroxy-5a-pregnane-20-one) was from Research Biochemicals (Natick, MA). Sodium PB was obtained from Gane's Chemical Works (Carlstradt, NJ). (+)-HX and (-)-HX were from Dr. J. Knabe (Saarbrucken, Germany). All other products were purchased from Sigma Chemical (St. Louis, MO) or as described in Methods.

Cell culture. Primary cultures of cerebellar granule cells were prepared from 6- to 8-day-old Sprague-Dawley rat pups as previously described (Iorio et al., 1992). In brief, cerebella were chopped using a McIlwain tissue chopper, and the tissue was treated with 0.25 mg/ml trypsin for 15 min at 37°C and dissociated by trituration. Cells were resuspended in basal essential medium containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 units/ml penicillin/100 μg/ml streptomycin and 25 mM KCl (Iorio et al., 1992). Cerebellar granule cells (3 × 10^6) were plated onto 100 × 15-mm culture dishes coated with poly-L-lysine and maintained at 37°C in 10% CO_2. The growth of non-neuronal cells was inhibited by culturing the cells in cytosine arabinofuranoside (10 μM), starting 24 hr after plating. Unless otherwise specified, on culture day 4, test drugs were added. On culture day 7, medium (with or without drugs) was removed; cells were washed three times in phosphate-buffered saline, scraped off and centrifuged at 1000 × g for 5 min. Cell viability was assessed as previously described (Iorio et al., 1993).

RNA isolation and cDNA synthesis. Total RNA from the cell pellet was extracted using the TRIzol reagent according to the manufacturer's instructions (GIBCO BRL, Gaithersburg, MD). First-strand cDNA synthesis reactions (500 μl) contained 1× reaction buffer (GIBCO BRL), 10 μg of total cellular RNA, 3.1 units of random hexamers (Pharmacia, Baie D'Urfe, Quebec, Canada), 10 units of RNasin (Promega, Madison, WI), 10 mM DTT, 2000 units of MMLV reverse transcriptase (GIBCO BRL) and 0.5 mM nucleotide triphosphates (Pharmacia). For an estimation of yield 9.5 μl of the reaction mixture was removed, and 0.5 μl of [35S]dCTP (Amersham, Oakville, Ontario, Canada) was added. The reaction mixtures were incubated in parallel at 37°C for 2 hr. [35S]dCTP incorporated into cDNA was separated from free labeled nucleotide by NICK column (Pharmacia) chromatography. The yield of cDNA was calculated after liquid scintillation counting. Control blanks for cDNA synthesis contained no RNA.

PCR amplification. For PCR amplifications, samples (100 μl) were brought to final concentrations of 10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM MgCl_2, 0.5 mM nucleotide triphosphate, 4% DMSO, containing 50 pmol of both the forward and reverse primers, 30 ng of cDNA template and 1.0 unit of Taq 1 polymerase (Perkin-Elmer Cetus, Norwalk, CT). An initial amplification cycle was run that consisted of denaturing at 98°C for 1 min, annealing at 55°C for 2 min and extending at 72°C for 2 min. PCR was then carried out for 20 to 50 cycles; each cycle consisted of denaturing at 94°C for 45 sec, annealing at 55°C for 60 sec and extending at 72°C for 60 sec. Primer sequences and verification of DNA products have been previously described (Tyndale et al., 1994). Oligonucleotide primers for gamma-2 are located at 1048 and 1358 nt, surrounding the 24-nt exon that produces the gamma-2L (311 nt, if present) or gamma-2S (287 nt, if absent) mRNA. After PCR, the DNA products were analyzed by electrophoresis in 1% agarose and 2% NuSieve GTG agarose (FMC) gels containing 0.5 μg/ml ethidium bromide. The gamma-2L and gamma-2S forms can be detected and measured on the ethidium bromide-stained gels or submitted to Southern blotting using [32P]-labeled probes. Because PCR can detect low levels of contaminating cDNA, RNA or genomic DNA sequences, we used multiple controls. (1) Water blanks were taken through the cDNA synthesis steps as negative controls to determine the cDNA synthesis did not introduce contamination. (2) RNA (no cDNA synthesis step) and water blanks were used to determine both contamination by cDNA or genomic DNA (detectable as each primer pair was designed to surround an intron resulting in genomic DNA PCR products of a larger-than-expected product size). (3) Samples without primers were run to detect primer contamination of template or reagents. (4) Rat brain cerebellar RNA positive controls were run for each experiment because the cells are derived from rat. (5) RT-PCR with NNE primers (Tyndale et al., 1994) was used to test whether cDNA synthesis was successful and to control for variation among samples.

Southern blotting. Electrophoretically separated DNA products were denatured, transferred to Zeta-Probe membranes (BioRad, Mississauga, Ontario, Canada) and prepared for screening according to the manufacturer's instructions. The membranes were hybridized at 42°C with [32P]dATP random primed cDNA probes. Exposure times varied from 10 min to 1 hr.

Quantification. The amount of product was measured with an image analyzer from Imaging Research (St. Catherine's, Ontario, Canada). Imaging was performed on the ethidium bromide-stained gels using a ultraviolet light source and/or on autoradiograms of Southern blots. In addition, radioactively labeled bands were excised from the radioactively probes Southern blot membranes and subjected to liquid scintillation counting.

Initially, we determined conditions under which PCR product formation from rat GABA_A receptor subunit gamma-2 cDNA templates (long or short) was log-linear. PCR amplification (28 cycles) of cDNA from gamma-2 plasmid templates (0.5–20 pg) was log-linear for both templates. When the plasmids were added in defined ratios (gamma-2L/S 0.05–1.7) that included the ratios detected in this study (see fig. 4B), detected ratios were equal to the ratio of cDNAs added to the reaction. Three methods were used for measuring the gamma-2L and gamma-2S DNA products from cells with and without PB treatments (i.e., imaging of agarose gels and Southern blots, or liquid scintillation counting). All three methods resulted in similar (no significant difference between methods) determinations of both high gamma-2L/S ratios (0.7, untreated control cells) and low gamma-2L/S ratios (0.3, PB-treated cells). Detection of PCR product was linear over multiple PCR cycles; the range depended on the sensitivity of the detection method. Using the Southern blotting method, detection of PCR product was linear for 20 to 30 PCR cycles, whereas detection from imaging of agarose gels was linear for 26 to 40 cycles. Detection and quantification of PCR product were performed by both of these two methods for each experiment using 28 cycles of PCR.

Cerebellar granule cell treatments. Unless otherwise specified, on culture day 4 the following drug treatments were initiated: RNA extraction occurred on day 7:

PB dose response. For dose-response determinations, 100, 250 and 500 μM (final concentration) PB was added to the medium. In all other experiments, 500 μM PB was used.

Time course studies. The time course for the action of PB was studied in three different paradigms. In the first two, the total number of days in culture was the same (7 days), whereas in the third, the total number of days in culture, with and without PB, varied (5–7 days in culture). In time course I, PB (500 μM) was added on day 4 (resulting in 3 days of PB treatment), day 5 (resulting in 2 days of PB treatment) or day 6 (resulting in 1 day of PB treatment). On culture day 7, total cellular RNA was extracted. In time course II (reversal of PB effect), PB was added to the cells on culture day 4 and...
replaced with medium on day 5 (1 day of PB treatment), day 6 (2 days of PB treatment) or day 7 (3 days of PB treatment). Removal of PB consisted of washing the cells three times in PBS and replacing the medium with conditioned medium without PB. Controls were included for each treatment to assess the effect of changing the medium on the different days. All extractions were done on culture day 7. In time courses I and II, the control ratio was not significantly altered by changing of the medium (to coincide with changing the medium of the PB-treated cells). Therefore, the control data from the individual experiments were pooled. In time course III, PB was added to the medium on culture day 4, and the cells, with or without PB (controls), were extracted on day 5 (1 day of PB treatment and control), day 6 (2 days of PB treatment and control) or day 7 (3 days of PB treatment and control). Thus, in this experiment the time of addition of PB was kept constant, but the extraction and isolation of RNA were carried out on different days, resulting in different numbers of total days in culture, with or without PB.

Other GABA_A receptor agonist studies. Cerebellar granule cells were treated chronically with ethanol (100 mM) for 3 days as previously described (Iorio et al., 1992). In brief, ethanol (5.85 µl of 100% ethanol/mL of medium) was added to the medium on culture day 4 to obtain a final concentration of 100 mM ethanol. The medium was supplemented daily with 3 µl of 100% ethanol/mL to maintain the proper concentration (Iorio et al., 1992). The cultures treated with ethanol were maintained within a larger dish containing 100 mM ethanol to reduce the loss of ethanol due to evaporation from the medium. The cells were extracted on culture day 7. Both isomers of HX (500 µM) and 5α-pregnane-3α,16α-diol-20-one, at two concentrations (1 and 2 µM final concentration), were also tested using a similar paradigm (i.e., addition of drugs on culture day 4, RNA extraction on culture day 7; however, in contrast to ethanol treatments, these drugs were added only once to the cells).

GABA_A receptor blocker studies. The ability of PX to block the effect of PB was examined by treating cells with 10 µM PX, alone and in combination with PB. PX and PB treatments were started on culture day 4 (3-day treatments) or 6 (1-day treatments).

Data analysis. Each set of coded samples contained a control sample plus a group of treatment samples (e.g., control and three different PB doses). Each set (two to five sets per experiment) was assayed by RT-PCR (three to 10 times per sample) for gamma-2L/S mRNA, resulting from the removal of the extra exon in the gamma-2L transcript.

PB treatments resulted in a profound decrease in the gamma-2L/S GAB_A receptor subunit mRNA ratio. The effect of PB treatments on the ratio of gamma-2L mRNA to gamma-2S mRNA is illustrated in figure 1. The addition of NNE control primers did not alter the results, and therefore NNE mRNA could be used as an internal control (data not shown). After adjusting for equal amounts of cDNA template in the PCR reactions (using NNE levels), the loss in gamma-2L was 4.83 ± 0.94, and the increase in gamma-2S was 4.59 ± 0.54, resulting in a gain of gamma-2S that was 99.9 ± 9.4% of the loss of gamma-2L. This strongly suggests that the loss of gamma-2L mRNA equals the gain in gamma-2S mRNA, resulting from the removal of the extra exon in the gamma-2L transcript. The control and PB-treated samples in figure 1 have gamma-2L/S ratios (±S.E.M.) of 4.46 ± 0.65 and 1.64 ± 0.036, respectively, a percent decrease by PB treatment of 64 ± 3%. The PB effect demonstrated a concentration-response relationship, with significantly greater changes in the gamma-2L/S mRNA ratio with increasing PB concentrations (fig. 2, table 1). The ratio was decreased by 15% with 100 µM PB, whereas 250 µM PB caused a greater decrease of ~40%, and 500 µM PB decreased the ratio by ~55% (significantly greater than either of the two lower concentrations). All of these decreases were statistically significant (P < .001) compared with control.

Time course for effect of PB on the gamma-2L/S mRNA ratio. In time course I, cells were treated with PB for 1, 2 or 3 days, with drug treatments starting on different days and ending on culture day 7 (fig. 3A). These results

**Results**

Validation of the methodology. Viability of the cells was unaffected by drug treatments as indicated by no changes in cell number, cell death, cell morphology or amount of extracted RNA. Cell viability, as judged under phase contrast, was not affected by any of the drug treatments. In addition, cerebellar granule cell survival was measured; cells exposed to PB or ethanol were not significantly different from controls (~100% of control values) with respect to cell survival (Iorio et al., 1993). This is a relatively homogeneous preparation of cerebellar granule cells (>97% of cells in the culture), a cell type with relatively homogeneous pharmacology. Therefore, changes in the gamma-2L/S mRNA ratio are unlikely to reflect cell death, migration or cell survival changes that are possible (likely) in vivo.

**A. Non-neuronal enolase**

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**B. Gamma 2**

**Fig. 1.** RT-PCR Southern blot from control and PB treatments. Southern blot of RT-PCR from a pair of control (C) and PB-treated cell cultures. Samples were assayed with NNE primers and gamma-2 primers. Adult cerebellar (CB) rat cDNA and RNA (BK) were used as positive and negative controls, respectively.

K. R. Short and B. Tabakoff, unpublished observations.
indicate that 1, 2 or 3 days of PB treatment each resulted in significant reductions in the gamma-2L/S mRNA ratio (50–60% decrease). Both 2 and 3 days of PB treatment caused statistically significant, if small, further decreases relative to 1 day of treatment.

In time course II, the PB treatment was started on culture day 4, and medium containing PB was replaced with control medium after 1, 2 or 3 days of PB treatment (fig. 3B). RNA was collected from all samples on day 7, so the total time in culture was equal for the treatments and controls. After 2 days without PB (i.e., the 1-day treatments), the ratio was not significantly decreased relative to control treatments, whereas after only 1 day without PB (i.e., the 2-day treatments), the decrease in the ratio was intermediate (40%) (fig. 4A). Two-day treatments (RNA harvesting on day 6) resulted in a significant reduction (60%) in the gamma-2L/S mRNA ratio, although not significantly more than the 1-day treatments. The 3-day PB treatments significantly decreased the ratio (70%) relative to control and 1 and 2 days of treatment.

The gamma-2L/S mRNA ratios in untreated control cultures were significantly different on culture days 6 and 7, suggesting that the ratio of gamma-2L/S mRNA is changing with time in culture (fig. 4B). The change in ratio may reflect in vivo developmental changes that are maintained during culturing in vitro or be a result of culturing of the cells. The change in gamma-2L/S mRNA ratio has been observed before, both in vivo and in culture (Bovolin et al., 1992), suggesting that it is most likely reflecting the in vivo situation. Data from time course III suggest that in fact, the gamma-2L/S ratio is responsive to PB on earlier days of culture (culture days 4 and 5), supporting the hypothesis that there was recovery of the gamma-2L/S mRNA ratio after removal of PB (time course II, fig. 4).

Ethanol, 5α-pregnan-3α-ol-20-one and HX treatments produced only minor changes in the gamma-2L/S mRNA ratio. We tested three additional GABA A receptor activators to determine the role of GABA A receptor activation in decreasing the gamma-2L/S mRNA ratio (fig. 5A, table 1). Ethanol (100 mM) had no effect on the gamma-2L/S ratio, whereas HX treatments [+]- and (-)-stereoiso-

### TABLE 1
Percent of control gamma2 L/S ratio of the drug treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent of control gamma2 L/S ratio</th>
<th>S.E.M.</th>
<th>Treatment</th>
<th>Percent of control gamma2 L/S ratio</th>
<th>S.E.M.</th>
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<tr>
<td>Time course I</td>
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<td>GABA A receptor agonist treatments</td>
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<td>1-Day PB</td>
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<td>3.3</td>
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<tr>
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<td>3.8</td>
<td>Ethanol 100 mM</td>
<td>82.2</td>
<td>2.4</td>
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<tr>
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<td>38.7</td>
<td>4.6</td>
<td>(+) HX 500 μM</td>
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<tr>
<td>2L/S mRNA ratio</td>
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<td>1.4</td>
<td>Allopregnanolone/ 1 μM</td>
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<tr>
<td>2L/S mRNA ratio</td>
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<td>1.4</td>
<td>Allopregnanolone/ 2 μM</td>
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<tr>
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<td>2</td>
<td>Allopregnanolone/ 2 μM</td>
<td>95.8</td>
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</table>

* P > .001 vs. control
* P > .01 vs. control
* P > .05 vs. day-1 or 100 μM PB.
* P > .01 vs. day-1 or 100 μM PB.
* P > .001 vs. day-2 or 250 μM PB.
* Allopregnanolone = 5α-pregnan-3α-ol-20-one.
mers; 500 μM PB for 1, 2 or 3 days by starting PB treatments on culture day 6, 5 or 4, respectively. RNA was extracted from all treatments on culture day 7. B, time course II: cells were treated with 500 μM PB for 1, 2 or 3 days with treatments starting on culture day 4 and ending on culture day 5, 6 or 7, respectively. RNA was extracted from all treatments on culture day 7. *** P < .001 vs. control; **** P < .001, ** P < .01 vs. 1 day; #### P < .001 vs. 2 days.

Fig. 3. PB treatments alter gamma-2L/S mRNA ratio: Time course effects. A, Time course I: cerebellar granule cells were treated with 500 μM PB for 1, 2 or 3 days by starting PB treatments on culture day 6, 5 or 4, respectively. RNA was extracted from all treatments on culture day 7. B, time course II: cells were treated with 500 μM PB for 1, 2 or 3 days with treatments starting on culture day 4 and ending on culture day 5, 6 or 7, respectively. RNA was extracted from all treatments on culture day 7. *** P < .001 vs. control; **** P < .001, ** P < .01 vs. 1 day; #### P < .001 vs. 2 days.

Fig. 4. Effect of the length of time in culture on PB alterations of the gamma-2L/S mRNA. PB and respective vehicle control (Con) treatments were started on culture day 4 (time course III). RNA was extracted on culture day 5 (1-day total duration), day 6 (2 days) or day 7 (3 days) in contrast to time courses I and II, in which the exposure to PB varied in duration, but the total time in culture before RNA extraction did not. A, Data as a percentage of each respective control gamma-2L/S ratio (e.g., plotted in the same manner as time courses I and II in fig. 3). B, Same data as absolute gamma-2L/S mRNA ratios. *** P < .001 vs. respective control; **** P < .001 vs. 1-day PB; #### P < .001 vs. 2-day PB; *** P < .001 vs. 1-day control; * P < .01 vs. 1-day control.

Blockade of the GABA_A receptor channel did not prevent PB from decreasing the gamma-2L/S mRNA ratio. To further assess whether GABA_A receptor activation was required for the decrease in the gamma-2L/S ratio, we treated cerebellar granule cells with PB in the presence or absence of the GABA_A receptor antagonist PX (10 μM; fig. 5B). Samples treated with PX alone showed no change in the ratio, indicating that receptor blockade per se did not alter the gamma-2L/S mRNA ratio. In addition, PX, when applied with PB, did not block the decrease in the gamma-2L/S ratio that was observed with PB alone.

Because of the possibility that PX was being degraded in the cultures, we also tested 1-day (culture days 6 and 7) treatments of PX with or without 1-day PB treatments. One-day treatments of PB effectively decreased the gamma-2L/S mRNA ratio, as shown previously (third day only, time course I), but PX was still unable to block the PB-mediated decrease in ratio. These results suggest that PB does not decrease the gamma-2L/S mRNA ratio through activation of the GABA_A receptor.

Discussion

The splicing of the GABA_A receptor subunit gamma-2 is affected by a barbiturate. In this study, we examined whether various GABA_A receptor agonists and an antagonist could alter the gamma-2L/S mRNA ratio. Decreasing the ratio could lead to decreased phosphorylation of the GABA_A receptor and/or altered gamma-2 conformation,
The barbiturate-mediated decreases in the gamma-2L/S mRNA ratio occur at concentrations that activate GABA$_A$ receptors. We have shown that the PB-mediated decrease in the ratio of gamma-2L/S mRNAs in cerebellar granule cell cultures occurs at concentrations (100–500 μM) that are sufficient and appropriate for affecting GABA$_A$ receptor function (10–100 μM) and binding (50–200 μM) (Huidobro-Toro et al., 1987; Leeb-Lundberg and Olsen, 1982; Slany et al., 1995). PB caused a decrease in the ratio of gamma-2L/S mRNAs in the current study, even though PB is a GABA$_A$ receptor agonist that does not require the presence of either the gamma-2L or gamma-2S subunit in the receptor complex (Günther et al., 1995; Sanna et al., 1995). The PB-mediated alterations in gamma-2L/S mRNA ratio was dependent on duration of PB treatment, were reversible and required as little as 1 day of PB treatment (fig. 3).

At equal concentrations (500 μM), (+)-HX was modestly more potent than (+)-HX in decreasing the gamma-2L/S ratio (30% vs. 20%) but far less potent than PB (70%). These HX stereoisomer effects are consistent with concentrations that activate GABA$_A$ receptors but not with the rank order of potencies for these two isomers at the GABA$_A$ receptor chloride channel as measured by binding or anesthetic threshold (Leeb-Lundberg and Olsen, 1982; Olsen et al., 1986; Wahlström, 1966). These data suggest that although both barbiturate compounds decreased the gamma-2L/S mRNA ratio, the actions are not mediated via GABA$_A$ receptor activation. 

Agonists, at concentrations that act at the GABA$_A$ receptor, do not affect the gamma-2L/S mRNA ratio. Some, but not all, previous studies have shown that ethanol activates GABA$_A$ receptors that contain the gamma-2L subunit, so a decrease in gamma-2L and an increase in gamma-2S (i.e., decreased gamma-2L/S ratio) could result in decreasing the actions of ethanol at the resultant receptors (Harris et al., 1995; Wafford et al., 1991; Wafford and Whiting, 1992; for a review, see Macdonald, 1995). On this basis, it would be reasonable to postulate that certain aspects of tolerance to the actions of ethanol on the GABA$_A$ receptor might involve the replacement of the gamma-2L subunit with the gamma-2S form (i.e., decreased gamma-2L/S ratio). Ethanol (30 mM) stimulates chloride flux in a microsac preparation by 50%, although there is no effect of ethanol on ligand binding to the GABA$_A$ receptor at concentrations up to 100 to 200 mM (Harris et al., 1995; Huidobro-Toro et al., 1987; Mehta and Ticku, 1988; Morrow et al., 1988). The decrease in gamma-2L/S ratio that was seen with PB treatment cannot be mimicked by ethanol at concentrations (100 mM) believed to be effective at the GABA$_A$ chloride channel.
receptor (fig. 5A, table 1). Similarly, 5α-pregnane-3α-ol-20-one, a potent GABA_A receptor agonist, did not alter the gamma-2L/S ratio (fig. 5A) at concentrations (1–2 μM) that are effective in altering GABA_A receptor function (0.2–6 μM; Gee et al., 1988; Morrow et al., 1990; Vincens et al., 1995). These cells have a mixture of GABA_A receptors and respond to a variety of agonists; therefore, it is unlikely that the lack of change after treatment with these agonists is due to a lack of receptor interaction with the agonists.

Decreases in the gamma-2L/S mRNA ratio are not mediated by GABA_A receptor occupation. To further confirm that the PB-mediated decrease in the gamma-2L/S ratio was not mediated by activation of the GABA_A receptor, we tested the ability of PX (a GABA_A receptor channel blocker) to alter the gamma-2L/S ratio. The fact that PX itself had no effect on the gamma-2L/S ratio, and was unable to block the decrease in ratio mediated by PB, supports our other findings of the lack of involvement of GABA_A receptor activation in the actions of PB on the gamma-2L/S ratio.

A study using an electrical kindling model of focal epilepsy found decreases in the total gamma-2 and the gamma-2L form (Kamphuis et al., 1995). The decrease in gamma-2L mRNA subunit was maintained on a long-term basis. This suggests that in addition to drug-mediated mechanisms, other mechanisms for altering the gamma-2 subunit exist.

The consequence of chronic barbiturate treatment on GABA_A receptors has been studied in terms of binding, chloride flux, development of tolerance and alterations in allosteric interactions (Allan et al., 1992; Liljequist et al., 1984; Möhler et al., 1984; Saunders et al., 1990, 1992; Suzuki et al., 1995; Tseng et al., 1993; Yu and Tichu, 1995). For example, chronic (5-day, 200 μM) PB treatment of mouse cortical neurons was found to decrease the efficacy of GABA, PB and neurosteroids for increasing [3H]flunitrazepam binding, whereas the potency values were unchanged (Yu and Tichu, 1995). The authors suggested that chronic PB treatment produced heterologous uncoupling of the GABA/benzo-diazepine receptor ionophore complex. Our results suggest that some of the effects observed after chronic barbiturate exposure may be mediated in part by alterations in the presence and phosphorylation of the GABA_A receptor gamma-2L and gamma-2S subunits, in addition to possible direct effects on the receptor.

One possible mechanism for barbiturate-mediated alterations of the gamma-2L/S ratio, not involving the GABA_A receptor, might be regulation of the spliceosome. These protein/RNA/ribonucleoprotein particle complexes are involved in the splicing of precursor mRNA to the mature mRNA form (for a review, see Draper, 1995). These complexes play a role in regulation of the inclusion or exclusion of exons, like the exon found in the gamma-2L mRNA (Kanopka et al., 1996). An effect like the one observed here may not be restricted to the central nervous system. Splicing of the drug-metabolizing enzyme CYP2B2 precursor RNA may also be affected by barbiturates (Lacroix et al., 1990). In that study, a CYP2B2 mRNA, with the addition of a novel 24-nucleotide exon, was observed in PB-induced rat liver. We were unable to identify any studies in which the specific effects of barbiturates on spliceosomes had been tested. The mechanism may be as simple as barbiturate induction of a required protein factor or an effect on splicing catalysis (Mermoud et al., 1994). These data suggest that there may be a general effect of barbiturates on the splicing of exons of multiple precursor RNAs.

Conclusions. In this study we demonstrated for the first time that barbiturate treatments could decrease the GABA_A receptor subunit gamma-2L/S mRNA ratio but that this effect was not mediated via direct activation of the receptor itself. Neither the mechanism nor the impact of decreasing the gamma-2L/S mRNA ratio is known, but changes in the ratio could produce decreased phosphorylation of the GABA_A receptor with subsequent alterations to GABA_A receptor function.

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References

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