Thiopental and Methohexital Depress Ca²⁺ Entry into and Glutamate Release from Cultured Neurons

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Background: Although barbiturates activate α-aminobutyric acid type A receptors as part of their hypnotic effect, these drugs also inhibit voltage-gated calcium channels. The authors determined if barbiturates could decrease neuronal intracellular Ca²⁺ transients and the resulting glutamate release.

Methods: Neonatal rat cerebellar granule neurons were isolated and cultured on coverslips and studied at 37°C. Spectrofluorometric assays were used during identical conditions to monitor intracellular Ca²⁺ with the Ca²⁺-sensitive fluorophore fura-2 and glutamate release by a glutamate dehydrogenase-coupled assay, which produced the reduced form of nicotinamide-adenine dinucleotide phosphate in proportion to the amount of glutamate released. Neurons were depolarized by a rapid increase in external [K⁺] from 5 to 55 mM. Control responses were compared with those in the presence of 10, 30, and 100 μM thiopental; 3, 10, and 30 μM methohexital; decreased external [Ca²⁺]; or voltage-gated calcium channel blockers.

Results: Thiopental and methohexital depressed the intracellular Ca²⁺ transient peak and plateau in a dose-dependent manner, as did decreased Ca²⁺. The intermediate dose of either drug caused ≈50% decrease in peak intracellular Ca²⁺ and 60% decrease in glutamate release. In the presence of specific L- and/or N-type voltage-gated calcium channel blockade by nicardipine or ω-conotoxin-GVIA, respectively, 30 μM thiopental further decreased the intracellular Ca²⁺ transient. Thiopental caused a dose-dependent decrease in glutamate release, which was proportional to the decreased peak intracellular Ca²⁺.

Conclusions: Thiopental and methohexital depress the depolarization-induced increase in intracellular Ca²⁺ and the accompanying glutamate release, actions which can contribute to the anesthetic and neuronal protective effects of these drugs. (Key words: Anesthetic mechanisms; barbituates; calcium channels; calcium; neural protection; synaptic transmission.)

THE anesthetic potency of the barbiturate class of sedative hypnotic agents has long been recognized. A major site of action of these drugs regarding their sedative hypnotic effects is related to their binding to the γ-aminobutyric acid type A (GABA$_A$) receptor–chloride channel complex.[1–4] A variety of studies have demonstrated, however, that in addition to effects on the GABA$_A$ receptor, barbiturates also modulate several other ion channels,[5,6] including voltage-gated neuronal calcium channels.[4,7–9] Granule neurons are the most common neuron in the central nervous system,[10] and those isolated from the cerebellum have been used widely in isolated culture for toxicologic and pharmacologic investigation as a well-established, uniform model of a neuronal cell.[11,12] In cerebellar granule (CG) neurons, influx of Ca²⁺ through a variety of types of voltage-gated calcium channels (VGCCs)[12–16] leads to an increase in intracellular Ca²⁺ concentration ([Ca²⁺]$_i$)[12] and release of the excitatory neurotransmitter glutamate.[17–20] Using CG neurons, we examined the effects of clinical concentrations of the rapidly acting barbiturates thiopental and methohexital on the depolarization-induced increase of [Ca²⁺]$_i$ and the resulting glutamate release. Drug actions were compared with those observed with altered extracellular [Ca²⁺] and with blockade of specific VGCCs.

Methods

Cell Isolation and Culture

Cerebellar granule neurons were prepared using a modification of the method of Novelli *et al.*[11] Using a protocol approved by the University of Virginia Animal Research Committee, cerebella were dissected from 10–14 Sprague-Dawley rat pups 5–7 days old. The tis-

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sues were cross-chopped into 0.3-mm^2 blocks and suspended for 45 min at 37°C in solution A, which contained 0.25 mg/ml trypsin type III and (in mM) 140 Na$^+$, 5.4 K$^+$, 1.2 Mg^{2+}, 140 Cl$^-$, 1 H$_2$PO$_4^-$, 14.5 glucose, 25 HEPES, and 0.3% bovine serum albumin, adjusted to *p*H 7.4. After 45 min, DNase I and trypsin inhibitor were added, and the suspension was gently centrifuged for 2 min. The supernatant was discarded, and the pellet was triturated 70 times in solution A without trypsin. After 5 min, MgCl$_2$ (to 2.5 mM) and CaCl$_2$ (0.1 mM) were added to solution A, and the cell suspension was collected, filtered through 70-μm mesh, and recentrifuged for 2 min. Neurons (2×10^6) from the resuspended pellet (2×10^6) were plated onto glass coverslips (11×22 mm, coated with poly-L-lysine or lightly etched with 10 M NaOH) in culture dishes. Neurons were cultured in basal Eagle's medium with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 μg/ml gentamicin, and 25 mM KCl, a partially depolarizing medium which stabilizes growth of CG neurons and prevents apoptosis. Proliferation of glial cells was prevented by addition of 10 μM cytosine arabinoside 24 h after plating. Cerebellar granule neurons were maintained in 5% CO$_2$/95% air at 37°C and were used at 4–8 days in culture. At the time of study, 7- to 10-μm neurons were clustered in 30- to 70-μm aggregations of cells, with "cables" forming connections between the scattered cell aggregates, a pattern identical to that demonstrated previously.[21]

Biochemical reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated. Appropriate aliquots of 100 mM thiopental (Gensia Labs, Ltd., Irvine, CA) or 100 mM methohexital (Eli Lilly, Indianapolis, IN) as the Na$^+$ salt were added to the solutions 5 min before depolarization by addition of KCl.

Measurement of Intracellular Calcium Concentration in Cultured Granule Neurons

Cytosolic [Ca^{2+}]$_i$ was measured using fura-2. Neurons on coverslips were incubated at 37°C for 20 min in basal medium containing 3 μM fura-2-AM (Molecular Probes, Eugene, OR), 16 μM bovine serum albumin, and (in mM) 155 Na$^+$, 5.0 K$^+$, 155 Cl$^-$, 5 HCO$_3^-$, 1 H$_2$PO$_4^-$, 1.25 Mg^{2+}, 1.2 Ca^{2+}, 5 glucose, and 20 N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), adjusted to *p*H 7.4. In some experiments, HEPES was substituted for TES with no alteration in behavior. After washing the neurons twice in fura-2–free solution, coverslips were inserted into a holder and placed in a cuvette containing 2 ml of solution; the neurons were then washed twice more with 2 ml of fresh medium. Measurements of [Ca^{2+}]$_i$ were performed at 37°C in a PTI luminescence spectrofluorometer (Photon Technology International, Monmouth Junction, NJ) equipped with a thermostatically warmed cuvette and magnetic stirrer. Fluorescence at 510 nm was determined for alternating excitation wavelengths of 340 and 380 nm; ratios were collected every 0.33 s for 1.5 min. Influx of Ca^{2+} was initiated by rapid addition of 100 μmol of KCl (in 33 μl) to achieve a final concentration of 55 mM. Calibration was performed by obtaining minimum and maximum fluorescence values using 10 μM ionomycin for maximum (Ca^{2+}-saturated) values and 5 mM EGTA for minimum values, respectively, for each coverslip. After calibration, [Ca^{2+}]$_i$ was calculated according to the standard formula of Grynkiewicz *et al.* using a Ca^{2+}-fura-2 dissociation constant of 224 nM,[22] using PTI software (Felix™; Photon Technology International) configured for the analysis. The computed value of [Ca^{2+}]$_i$ was displayed and stored for subsequent analysis.

Measurement of Glutamate Release

Glutamate release was measured by a glutamate dehydrogenase–coupled assay. A coverslip with adherent CG neurons was rinsed with buffer solution and then placed in a cuvette containing 2 ml of buffer solution, which contained 1 mM of the oxidized form of nicotinamide-adenine dinucleotide phosphate (NADP+) and 50 U/ml glutamate dehydrogenase (Boehringer Mannhein, Indianapolis, IN, GmbH, Germany) to catalyze from the released glutamate the formation of α-ketoglutarate and the fluorescent species of the reduced form of NADP (NADPH), as previously described.[20,23] NADPH fluorescence was excited at 340 nm and measured at 460 nm using the PTI spectrofluorometer. Glutamate release was again activated by rapid addition of 100 μmol KCl for a final [K$^+$] of 55 mM, and the change in NADPH fluorescence was monitored for 5 min at a sampling rate of 1–2 Hz.

To calibrate the fluorescent response to glutamate release, studies were performed with the direct addition of NADPH or glutamate during identical conditions. Addition of NADPH in the cuvette solution to obtain concentrations of 0.2, 0.5, and 1.0 μM resulted in abrupt increases in the fluorescence signal of 0.92 ± 0.21, 1.94 ± 0.86, and $3.1 \pm 1.31 \times 10^5$ counts/s, respectively (mean $\pm$ SD, n = 5). When glutamate was added (as aliquots of 0.5 mM solution) to solutions containing 50 mg/ml glutamate dehydrogenase enzyme solution, the
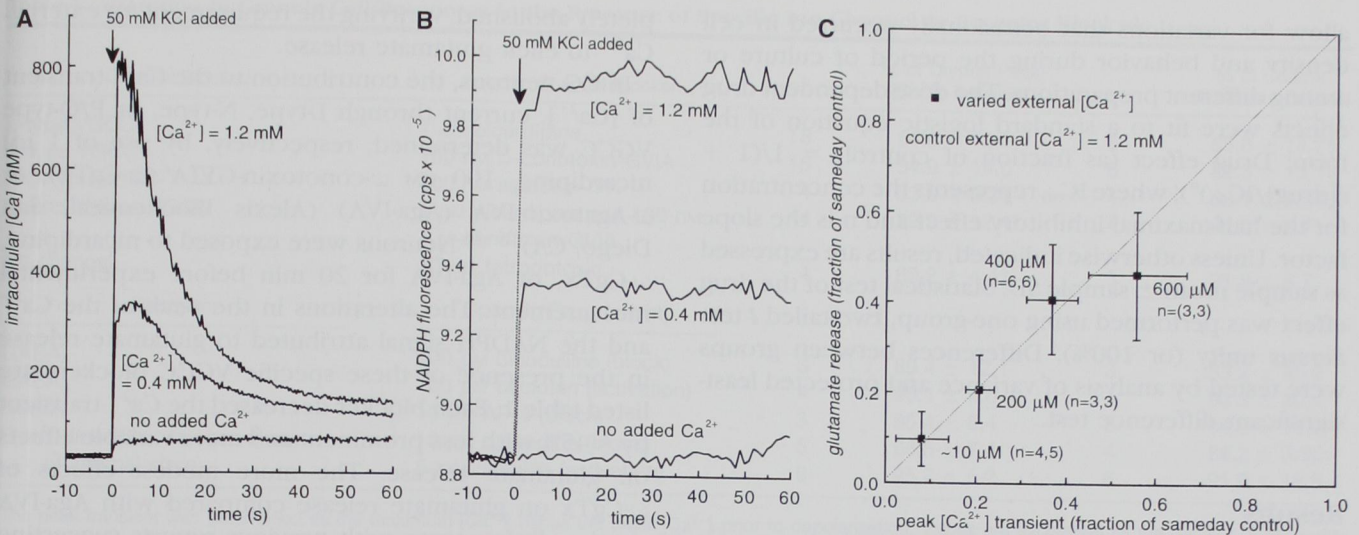
Fig. 1. Intracellular $[Ca^{2+}]$ transients in and glutamate release from cultured neonatal rat cerebellar granule (CG) neurons on a coverslip. Influx of Ca^{2+} and glutamate release were elicited by the abrupt increase of the external $[K^+]$ from 5 to 55 mM by the addition of KCl to a stirred solution in the cuvette. Tracings are shown for studies performed in normal extracellular $[Ca^{2+}]$ of 1.2 mM, 0.4 mM, and no added Ca^{2+} (free $[Ca^{2+}]$ was $\approx$10 mM). (*A*) Intracellular $[Ca^{2+}]$ concentration ($[Ca^{2+}]_i$) was determined in fura-2–loaded cells using the excitation fluorescence ratio method, in which calibration of the ratio was performed by subsequent cell permeabilization (sample rate = 3 Hz). (*B*) Glutamate release estimated by the fluorescence of NADPH, which is produced from NADP+ in proportion to the amount of glutamate released in the presence of glutamate dehydrogenase (sample rate = 1 Hz). (*C*) Relation between the peak Ca^{2+} transient and glutamate release in varied external $[Ca^{2+}]$. Results are expressed as the fraction of same-day control; the points and error bars represent the mean $\pm$ SD. The n values for the peak $[Ca^{2+}]_i$ and glutamate, respectively, are given in parentheses. The dotted line represents the line of unity, *i.e.,* equal changes.

fluorescence signal increased with an exponential time course time constant of $\approx$60 s at 37°C. When the added [glutamate] was 0.2, 0.5, and 1.0 μM (equimolar to the increases in [NADPH]), the respective steady-state increases in fluorescence signals were 0.89 $\pm$ 0.19, 1.83 $\pm$ 0.29, and 2.89 $\pm$ 0.30 $\times$ 10^5 counts/s, or $\approx$95% of the NADPH values. The close agreement of the fluorescence signal between the same quantity of NADPH and glutamate suggests that the glutamate reaction producing NADPH proceeded largely to completion.

With the addition of KCl and the depolarization of neurons grown on coverslips, there was a sudden increase in the fluorescence signal, often followed by a much smaller and slower increase, which typically stabilized by 5–15 s (fig. 1B). The increase in the fluorescence signal was typically on the order of 0.4–1.5 $\times$ 10^5 counts/s, suggesting an immediate glutamate release of 0.1–0.2 nmol. The value varied with the degree of confluence and coverage of neurons on the coverslips. Compared with the addition of glutamate in solution, the stabilization of the fluorescence signal in the presence of depolarization-induced glutamate release from neurons was more rapid. Such rapidity suggests that there must be rapid release of a high concentration

of glutamate, followed by the rapid arrest ($\leq$1 s) of glutamate release and local uptake of glutamate into neurons to cause cessation of production of NADPH in the first few seconds. A high concentration of glutamate ($>$100 μM) has been predicted in synaptic clefts,[24] whereas a high-capacity system for uptake of glutamate present in neurons could account for the rapid stabilization of the signal.[25]

Analysis and Statistics

To reduce artifacts attributable to inherent noise in the fluorescence signal, $[Ca^{2+}]_i$ was determined by averaging the individual values obtained during a 10-s period before depolarization (basal), during the 1–3 s immediately after depolarization (maximum), and during the final 5 s of the sample (plateau). The peak $[Ca^{2+}]_i$ transient is reported as the maximum value achieved minus the initial baseline value, although results did not differ if instead the actual maximum $[Ca^{2+}]_i$ was used for the calculation. NADPH fluorescence was calculated similarly except that a 1- to 5-s sample after depolarization was used and the plateau value was not determined. For comparison among various concentrations, results were expressed as the fraction of same-day control to

allow for variations that occasionally occurred in cell density and behavior during the period of culture or among different preparations. The dose-dependent drug effects were fit to a standard logistic equation of the form: Drug effect (as fraction of control) = $1/(1 + ([drug]/IC_{50})^n)$, where IC_{50} represents the concentration for the half-maximal inhibitory effect and n is the slope factor. Unless otherwise indicated, results are expressed as sample mean ± sample SD. Statistical test of the drug effect was performed using one-group, two-tailed *t* test *versus* unity (or 100%). Differences between groups were tested by analysis of variance and protected least-significant difference test.

Results

Control Experiments

As shown by the control response in figure 1A, a sudden increase in $[K^+]$ to 55 mM caused an increase in $[Ca^{2+}]_i$ from ≈40 to >800 nM, which subsequently declined to less than one-half the peak value in <15 s. $[Ca^{2+}]_i$ typically reached final plateau levels of 160–210 nM by 60–90 s. To verify the relation between peak $[Ca^{2+}]_i$ transient and the fluorescence attributed to glutamate release, a series of experiments was performed in which extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_e$) was decreased to <1.2 mM. Omission of added external Ca^{2+} in the absence of added EGTA produced a solution in which residual trace $[Ca^{2+}]_e$ was typically 0.005–0.010 mM (measured by ion-selective electrode). With $[Ca^{2+}]_e$ of 0.60, 0.40, 0.20, and ≈0.01 mM, the $[Ca^{2+}]_i$ transient was reduced to values that were 56 ± 11, 37 ± 6, 20 ± 3, and 8.1 ± 5.8% of the same day control, respectively (n = 5, 3, 6, and 3, respectively). The plateau of the $[Ca^{2+}]_i$ transient showed slightly more modest decreases to 78 ± 33, 64 ± 18, 33 ± 8, and 32 ± 19% of control, respectively. For identical decreases in $[Ca^{2+}]_e$, glutamate release decreased to 46 ± 14, 41 ± 12, 21 ± 7, and 9.6 ± 6.0% (n = 7, 7, 7, and 4, respectively) of same day control. The decrease was very similar to that for the peak $[Ca^{2+}]_i$ transient. The Ca^{2+} transient and the NADPH fluorescence attributed to glutamate release are shown in figure 1 for 1.2 and 0.4 mM Ca^{2+} and for no added Ca^{2+}. Figure 1C shows the relation between the peak Ca^{2+} transient and glutamate release for the reduction in $[Ca^{2+}]_e$. With 1 mM EGTA and no Ca^{2+} added to the extracellular solution, $[Ca^{2+}]_e$ was reduced to <100 nM. The $[Ca^{2+}]_i$ transient and glutamate release activated by KCl were then com-

pletely abolished, verifying the requirement for external Ca^{2+} to elicit glutamate release.

In CG neurons, the contribution to the Ca^{2+} transient of $[Ca^{2+}]_i$ current through L-type, N-type, or P/Q-type VGCC was determined, respectively, by use of 1 μM nicardipine, 100 nM ω-conotoxin-GVIA (ω-CgTx), or ω-Agatoxin-IVA (Aga-IVA) (Alexis Biochemical, San Diego, CA).[26-28] Neurons were exposed to nicardipine, ω-CgTx, or Aga-IVA for 20 min before experimental measurement. The alterations in the peak of the Ca^{2+} and the NADPH signal attributed to glutamate release in the presence of these specific VGCC blockers are listed table 1. Each blocker decreased the Ca^{2+} transient by ≈45% with less prominent and more variable effects on glutamate release. The more modest effects of ω-CgTx on glutamate release compared with Aga-IVA or nicardipine agree with previous reports suggesting that N-type channels contribute modestly to glutamate release.[18,20] When combined, the nicardipine and ω-CgTx showed an effect greater than either drug alone, but the increase was nonadditive. Such overlapping drug sensitivity also has been previously reported.[29] In two experiments, all three agents were combined, and the residual Ca^{2+} transient was reduced to 16 and 21% of control, suggesting that the bulk of the Ca^{2+} transient was VGCC-mediated Ca^{2+} entry.

In theory, the KCl-induced depolarization should inactivate sodium channels after an initial opening (<10 ms) so that the Ca^{2+} transient should reflect Ca^{2+} entry mediated by opened VGCCs. To determine if any of the observed effects were mediated by actions on sodium channels, depolarization also was performed in the presence of 10 μM tetrodotoxin to block sodium channels. A small (≈10%) fraction of the Ca^{2+} transient was depressed by the presence of tetrodotoxin, and its action of similar magnitude inhibiting glutamate release did not achieve significance. To verify that the observed events were activated by depolarization, and not the 28% increase (+100 mOsm) in osmotic strength, 100 μmol of NaCl was added instead of KCl, with no effect on the $[Ca^{2+}]_i$ transients or on glutamate release (n = 3).

Additional control experiments were performed to exclude possible effects of barbiturates on other processes in CG neurons known to influence glutamate release and VGCC function, such as GABA$_A$, GABA type B (GABA$_B$), and *N*-methyl-D-aspartate (NMDA) receptor activation.[30,31] GABA$_A$ receptors (chloride ion channels) were blocked using 100 μM bicuculline; GABA$_B$ receptors were activated by 10 μM baclofen; and NMDA gluta-

Table 1. Alteration in Granule Cell Responses in the Presence of Specific Ion Channel or Receptor Blockade

Channel Blocked	Specific Channel Blocker	n	% of Control Peak $[Ca^{2+}]_i$ Transient	n	% of Control Glutamate Release
L-type VGCC	1 μM nicardipine	18	$54.6 \pm 13.0^*$	6	$68.6 \pm 14.2\dagger$
N-type VGCC	100 nM ω-conotoxin-GVIA	15	$54.9 \pm 14.6^*$	6	$82.3 \pm 13.7\S$
P/Q-type VGCC	100 nM agatoxin IVA	4	$53.9 \pm 8.3\dagger$	4	$63.1 \pm 15.8\ddagger$
L- and N-type VGCC	1 μM nicardipine and 100 nM ω-conotoxin-GVIA	14	$39.9 \pm 12.4^*$	4	$51.0 \pm 2.7^*$
Na channel	10 μM tetrodotoxin	4	$89.2 \pm 4.4\ddagger$	3	91.9 ± 5.7
Receptor	**Specific Drug (effect)**				
GABA$_A$	100 μM bicuculline (block)	3	89.4 ± 12.1	3	85.2 ± 25.9
GABA$_B$	100 μM baclofen (activation)	4	95.1 ± 4.0	4	93.3 ± 30.4
NMDA	10 μM AP-5 (block)	3	95.4 ± 6.4	3	104.0 ± 14.2
Ryanodine (Ca^{2+} release channel of internal store)	10 μM ryanodine (block)	5	95.5 ± 7.4	4	$86.2 \pm 3.3\S$
	5 mM caffeine (activation)	9	95.7 ± 5.2	6	91.8 ± 16.9

The peak transient was determined as the maximum $[Ca^{2+}]_i$ minus the basal $[Ca^{2+}]_i$ prior to depolarization. Values are expressed as the percent of same day control, mean $\pm$ SD.

Different from 100% by one group two-tailed t test: $^* P < 0.001$, $\dagger P < 0.01$, $\ddagger P < 0.02$, $\S P < 0.05$.

mate receptors were inhibited by D-(−)-2-amino-5-phosphonovaleric acid. None of these interventions had any significant action on the depolarization-evoked Ca^{2+} transient or glutamate release (table 1). To determine if Ca^{2+} release from intracellular stores (endoplasmic reticulum) contributed to the Ca^{2+} transient or glutamate release, intracellular Ca^{2+} release was manipulated by modifying the function of the ryanodine receptor (Ca^{2+} release channel). Neurons were incubated with 10 μM ryanodine or 5 mM caffeine for 20 min before depolarization. During these conditions, ryanodine caused a small decrease (14%) in glutamate release (table 1), suggesting that internal Ca^{2+} release could contribute modestly to glutamate release in this setting.

Effects of Barbiturates

Administration of thiopental or methohexital did not alter the basal $[Ca^{2+}]_i$ but did markedly depress the depolarization-induced influx of $[Ca^{2+}]_i$. In the presence of thiopental or methohexital (figs. 2A and 2B) the peak $[Ca^{2+}]_i$ transient phase was decreased in a concentration-dependent manner. When analyzed as the fraction of same-day controls, the decrease in the peak $[Ca^{2+}]_i$ transients by the varying concentrations of barbiturate were fit by a logistic equation (fig. 2C). The calculated IC$_{50}$ (mean $\pm$ SEM) for thiopental was 34.5 $\pm$ 4.5 μM, 3.7 times greater than the value for methohexital of 9.6 $\pm$ 2.0 μM. The plateau phase of the Ca^{2+} transient was also depressed by the barbiturates but typically not to the same extent as the peak $[Ca^{2+}]_i$.

Detailed studies of the effects of thiopental were performed in the presence of specific calcium channel blockade. In the presence of a near IC$_{50}$ concentration of thiopental of 30 μM, the $[Ca^{2+}]_i$ transient peak was depressed to 56 $\pm$ 9% of control (n = 8). A similar decrease to $\approx$55% of control was observed with 1 μM nicardipine, 100 nM ω-CgTx (n = 14), or 100 mM Aga-IVA (n = 4). Nevertheless, when 30 μM thiopental was present with 1 μM nicardipine or ω-CgTx, a greater decrease in the Ca^{2+} transient was observed than seen with complete blockade of either the L- or N-type channels (fig. 3). These results suggest that thiopental was depressing multiple types of calcium channels. Conversely, a combination of either 1 μM nicardipine or 100 nM ω-CgTx with 30 μM thiopental caused a significantly greater decrease in the $[Ca^{2+}]_i$ transient peak compared with thiopental alone, suggesting that this near IC$_{50}$ concentration of thiopental had not completely blocked either N- or L-type channels. When L- and N-type channels were blocked by the combination of nicardipine and ω-CgTx, the peak Ca^{2+} transient was decreased (40 $\pm$ 12% of control) more than with either agent alone ($P < 0.01$). When 30 μM thiopental was also present, there was further marked decrease in the transient (fig. 3). This result suggests that 30 μM thiopental is able to depress Ca^{2+} entry profoundly through the P/Q-type and R-type (residual or blockade-resistant) VGCCs that remain functional in the presence of nicardipine and ω-CgTx.
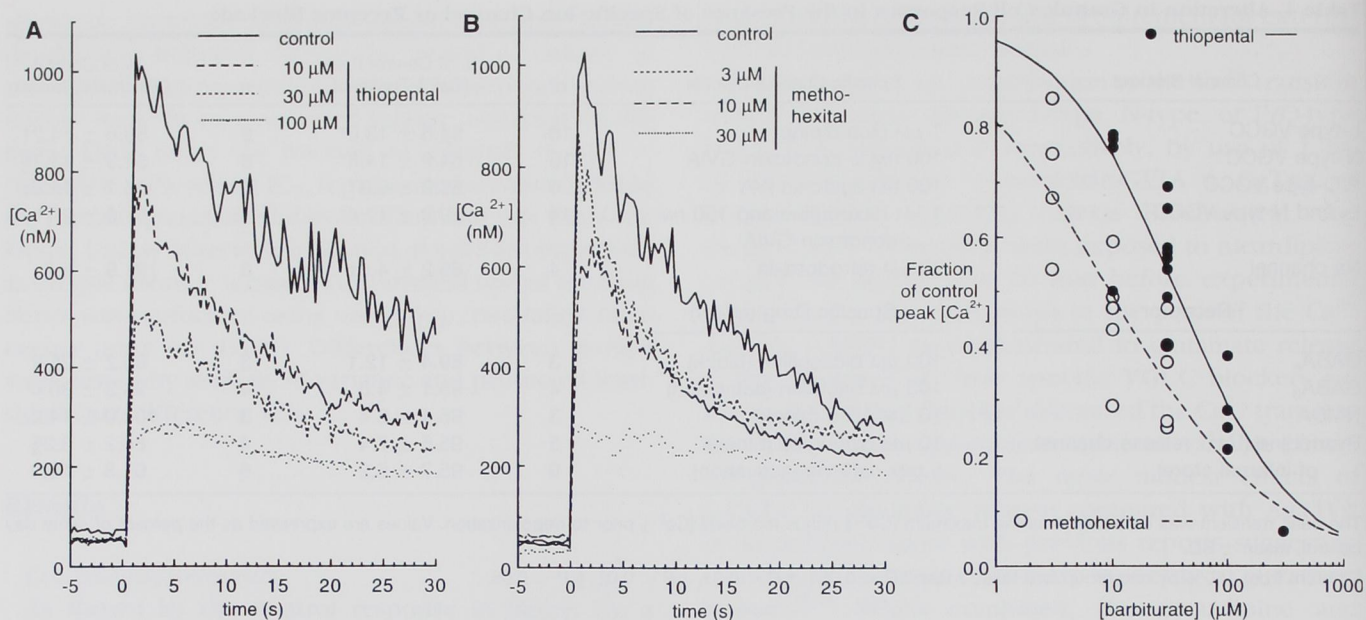
The inset in figure 4A shows the increase in NADPH

Fig. 2. Effects in cultured granule (CG) neurons of thiopental and methohexital on intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) transients elicited by a depolarization induced by sudden increase in $[K^+]$ from 5 to 55 mM at time zero. $[Ca^{2+}]_i$ was determined by the excitation fluorescence ratio method in cells that had been loaded with fura-2. Transients are shown for neurons on different coverslips from the same batch of cultured neurons on the same day. Responses are shown for control and in the presence of different concentrations of thiopental (*A*) or methohexital (*B*). (*C*) Dose dependence of decrease in the peak $[Ca^{2+}]$ transient by either thiopental (*filled circles*) or by methohexital (*open circles*). Each point represents a determination expressed as a fraction of the control measurement(s) made the same day. The lines represent a least-squares fit to the standard logistic equation: Fraction of control = $1/(1 + ([drug]/IC_{50})^n)$, with an IC_{50} (mean ± SEM) for thiopental of 36 ± 5 μM and slope factor n (mean ± SEM) of 0.85 ± 0.15. For methohexital, the IC_{50} value was 9.6 ± 2.0 μM, with n of 0.63 ± 0.15.
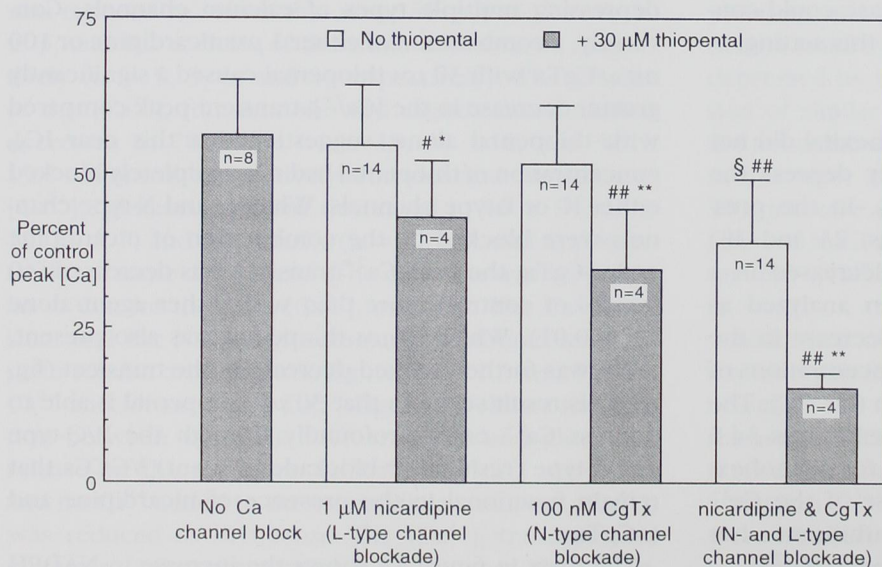

Fig. 3. The decrease in the peak Ca^{2+} transients elicited by depolarization with 55 mM KCl expressed as percent of same-day control. The mean decrease (±SD) caused by L-type calcium channel blockade with 1 μM nicardipine or 100 nM N-type calcium channel blockade with ω-conotoxin-GVIA (ω-CgTx) is shown, in the absence and presence of 30 μM thiopental. The inclusion of 30 μM thiopental with nicardipine, ω-CgTx, or their combination, resulted in further decrease in the Ca^{2+} transient. *$P < 0.05$ and **$P < 0.01$ for difference from setting with no thiopental; #$P < 0.05$ and ##$P < 0.01$ for difference from 30 μM thiopental with no calcium channel blocker; §$P < 0.5$ for difference from 1 μM nicardipine or 100 nM ω-CgTx alone.

Fig. 4. Effects of barbiturates on glutamate release from cultured CG neurons. (*A*) Dose-dependent decrease in glutamate release with thiopental, in which each point represents a determination expressed as a fraction of the control measurement(s) made the same day. The line represents a least-squares fit to the logistic equation: Drug effect = $1/(1 + ([drug]/IC_{50})^n)$, with an IC_{50} value for thiopental of 12.5 ± 1.4 μM and n of 0.68 ± 0.07. Neurons growing on a coverslip were depolarized by the sudden increase in $[K^+]$ from 5 to 55 mM at time zero, resulting in the sharp increase in NADPH fluorescence (see tracings inset, 1-Hz sample rate). Glutamate released in the presence of glutamate dehydrogenase results in generation of NADPH from NADP+ and glutamate. (*B*) Decrease in glutamate release plotted *versus* the decrease in the peak intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) transient (fig. 2C) determined during identical conditions. Effects of 10, 30, and 100 μM thiopental (*circles*) and 10 μM methohexital (*square*) are indicated, and the dashed line represents the line of unity, *i.e.*, equivalent decrease. Results are expressed as the fraction of same-day control; the points and error bars represent the mean ± SD. The n values for the peak $[Ca^{2+}]_i$ and glutamate, respectively, are given in parentheses.
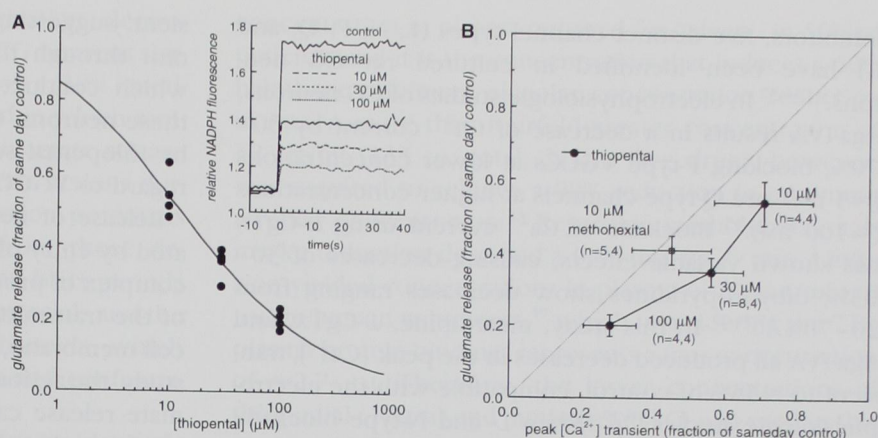
fluorescence observed with KCl depolarization, which represents formation of NADPH from glutamate and NADP+. Thiopental caused a dose-dependent reduction in glutamate release in these neurons (fig. 4A), which, by logistic analysis, was found to have an IC_{50} value of 12.5 ± 1.4 μM. In the presence of 10 μM methohexital, glutamate release was decreased to 41 ± 4% of same-day control, significantly less than the decrease to 54 ± 7% of control observed with 10 μM thiopental. To delineate more clearly the relation between effects on the peak $[Ca^{2+}]_i$ transient and on glutamate release, the average fractional decrease in glutamate release is plotted *versus* the average decrease in the peak $[Ca^{2+}]_i$ transient for the various barbiturate concentrations studied (fig. 4B). Clearly, there is a parallel decrease in the transient peak and glutamate release with either barbiturate, similar to the action observed in decreasing Ca^{2+} entry by reductions in $[Ca^{2+}]_e$.

Discussion

The current results have implications regarding mechanisms of anesthetic action and possibly the neuroprotection afforded by barbiturates during certain circumstances. Although it is clear that barbiturate activation of $GABA_A$ receptors/chloride channels mediates their hypnotic effect and may contribute to their anticonvulsant actions,[1-3,32] concentrations that produce anesthesia and lack of response to pain are typically higher.[33]

Previous studies have suggested the ability of barbiturates to inhibit Ca^{2+} currents[8,9,34,35] and the depolarization-induced increase in $[Ca^{2+}]_i$ at higher concentrations.[36] Macdonald *et al.* have suggested that for pentobarbital, although low concentrations can provide sedation, the higher concentrations required for complete anesthesia or coma are those that block calcium channels in neurons.[4]

Barbiturate-induced decrease in Ca^{2+} currents has been previously described in neurons[4,34,35] and cardiac myocytes[6] and for Ca^{2+} currents in *Xenopus* oocytes resulting from injection of brain-derived messenger ribonucleic acid.[9] The observed reduction in Ca^{2+} transients by barbiturates is consistent with a decrease in the influx of Ca^{2+} through VGCCs. Because KCl depolarization was unable to increase $[Ca^{2+}]_i$ in solutions that lacked Ca^{2+}, it is likely that KCl-induced increase of $[Ca^{2+}]_i$ resulted from the influx of Ca^{2+} through VGCCs. The action of barbiturates observed in this study is similar to the effect of methohexital on cultured embryonic rat hippocampal neurons reported by Bleakman *et al.*, in which 5–20 μM methohexital caused a 40–50% reduction in the peak of the KCl-induced Ca^{2+} transient.[36]

Cultured granule neurons have become a well-established model for the study of neuronal function, because their responses to various stimulatory paradigms are well described and their influx of Ca^{2+} has been studied by radioisotopic tracer, optical, and electrophysiologic methods. Using a series of specific VGCC

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inhibitors, five distinct channel types (L, N, P, Q, and R) have been identified in cultured rat CG neurons.[15,16,37] In electrophysiologic studies of CG neurons, Aga-IVA results in a decrease of Ca^{2+} current by 50–70%, blocking P-type VGCCs at lower concentrations (≈ 1 nM) and Q-type channels at higher concentrations (≈ 100 nM).[15] Blockade of Ca^{2+} current using ω-CgTx has shown variable effects, causing decreases of 30–63%; dihydropyridines show decreases ranging from 20–70%.[15,16,29] In this study, nicardipine, ω-CgTx, and Aga-IVA all produced decreases in the peak $[Ca^{2+}]_i$ transient to $\approx 55\%$ of control, compatible with the electrophysiologic studies.[16,29] When L- and N-type blockade were combined, the effect on the $[Ca^{2+}]_i$ transient peak was not strictly additive in that 40% of the control peak $[Ca^{2+}]_i$ was still present. Such nonadditive decreases have been previously reported in an electrophysiologic investigation and may represent overlapping sensitivity between the blockade by these two agents.[29] Nevertheless, the effect of the combined agents was greater than either agent alone, suggesting that overlap was incomplete. When L-, N-, and P/Q-type VGCCs are inhibited by combined application drug and toxins, the 19% of total Ca^{2+} current that remains has been attributed to the R-type channel.[15] Although some discrepancy between voltage clamp studies and the current measures of $[Ca^{2+}]_i$ would be anticipated based on the finding that the fluorometrically measured $[Ca^{2+}]_i$ may not be a linear function of Ca^{2+} currents, a surprisingly similar fraction of the $[Ca^{2+}]$ transient ($\approx 19\%$) remained when combined L-, N-, and P/Q-type blockade was used in this study.

To assess whether thiopental was decreasing Ca^{2+} influx by inhibiting specific VGCCs in these CG neurons, nicardipine (1 μM) and ω-CgTx (100 nM) were used at concentrations sufficient to provide complete blockade of L- and N-type channels, respectively.[20,29] These studies of nicardipine or ω-CgTx with 30 μM thiopental, which caused a similar decrease of peak $[Ca^{2+}]_i$, permit certain qualitative conclusions. Because either L- or N-type blockade did cause an additional decrease in $[Ca^{2+}]_i$ when combined with 30 μM thiopental, thiopental could not have been completely blocking either L- or N-type VGCCs. Conversely, the finding that thiopental by itself decreased the peak $[Ca^{2+}]_i$ to the same extent as nicardipine or ω-CgTx current suggests it must have been blocking more than one type of channel. After blockade of both L- and N-type VGCCs, the transient is reduced by 60%, yet addition of 30 μM thiopental causes an additional 25% inhibition of the Ca^{2+} tran-

sient, suggesting that thiopental is blocking Ca^{2+} current through the P/Q- and R-type calcium channels, which conduct the complement of Ca^{2+} current in these neurons. Consequently, the inhibition of VGCCs by thiopental seems to be relatively nonspecific with regard to VGCC type.

Release of neurotransmitters from neurons is mediated by entry of Ca^{2+} into nerve terminals, activating a complex of proteins that cause fusion of the membrane of the transmitter-containing synaptic vesicle with the cell membrane, resulting in exocytosis.[38] In the current study, the actions of the barbiturates in decreasing glutamate release can be attributed to their depression of VGCC-mediated entry of Ca^{2+}. In these CG neurons, as with other central synapses,[39] glutamate release does not appear to be associated with any specific calcium channel, as blockade of various VGCCs is capable of decreasing glutamate exocytosis from granule cells.[20] Voltage-gated calcium channel blockade of N-type channels using ω-CgTx, in some instances, has been found not to inhibit glutamate release[18,20]; however, the brief preincubation periods (5 and 8 min) used in these studies may not have been sufficient to observe the decrease in neurotransmitter release reported for other neurons.[39,40] In preliminary experiments, we found that ω-CgTx applied for 5 min provided only modest blockade of the $[Ca^{2+}]$ transient, whereas 20 min caused the greater blockade reported in table 1. The activation of release of internal Ca^{2+} stores by metabotropic glutamate receptors does not appear to occur in these neurons grown during these mildly depolarizing conditions.[21] Although certain components of entry of Ca^{2+} have been attributed to entry of Ca^{2+} *via* other pathways, such as NMDA receptors, block of NMDA receptors also did not have a prominent effect on the depolarization-evoked response in these cells in this study.

In the physiologic setting, when exocytosis is activated by action potentials, the transmitter release is reduced in proportion to some power function of the reduction in $[Ca^{2+}]$, suggesting there is cooperativity in Ca^{2+} dependence and that two or more Ca^{2+} must bind to specific sites.[39,41–43] In this study, the prolonged depolarization induced by KCl produced sustained rather than intermittent entry of Ca^{2+}, which may result in saturation of activating Ca^{2+} sites, resulting in the more linear relation between the peak Ca^{2+} and glutamate release (fig. 1C). In addition, the finding that the measured $[Ca^{2+}]_i$ may reflect $[Ca^{2+}]$ at other than intrasynaptic sites means that such quantitative correlations should be interpreted cautiously. Although the VGCC

inactivates during this sustained depolarization, electrophysiologic studies of Ca^{2+} currents in CG neurons suggest that inactivation is incomplete at 1 – 3 s (38% inactivation at 0.72 s),[37] which was the period in which sampling of the peak Ca^{2+} transient and glutamate release were performed. Nevertheless, barbiturate actions during physiologic conditions in which action potentials activate VGCC may differ markedly from those described here, because of the intermittent, brief periods of VGCC activity generated by action potentials and the effects of barbiturates on other ion channels, which could alter action potential conduction and configuration.

The $\approx$80% decrease in the Ca^{2+} transient by blockade of drug- and toxin-sensitive VGCCs (not including R-type VGCC) suggests the Ca^{2+} transient in this setting of KCl depolarization is derived largely from entry of Ca^{2+} through VGCC. Because the IC_{50} value for the effects of thiopental on glutamate release was approximately one third that for decrease in concentration, however, thiopental might alter other cellular pathways contributing to glutamate release. Exocytosis also may involve release of Ca^{2+} from internal stores in some instances. Although in cultured CG neurons G protein – linked receptor activation of intracellular Ca^{2+} can be altered by caffeine or ryanodine,[44] manipulation of internal Ca^{2+} release channels did not alter the depolarization-induced Ca^{2+} transient in this study. The modest action of ryanodine in modestly decreasing glutamate release suggests, however, that thiopental actions on internal Ca^{2+} release could contribute to its actions. A thiopental-induced decrease in release of $[Ca^{2+}]_i$ has been described in myocardium for the concentrations used in this study[45] and might explain the slightly greater decrease in glutamate release compared with the Ca^{2+} transient. Although glutamate is the primary excitatory transmitter in the nervous system, a previous study using striatal synaptosomes from rat found that thiopental also is able to depress a depolarization-induced increase in dopamine.[46]

The low and intermediate concentrations of barbiturates used represent concentrations of free (unbound) drug that may occur clinically. A 6-mg/kg intravenous bolus dose of thiopental results in a peak total serum concentration of 93 μg/ml,[47] whereas the average concentration of anesthetic agent in plasma is 12.6 μg/ml.[48] Assuming 83 – 86% plasma protein binding, the resulting free thiopental concentrations should be 7.2 – 60.0 μM (1.9 – 16.0 μg/ml). A concentration of thiopental of 7.3 – 7.4 μg/ml (31 μM total, 5 μM free drug) represents the concentration in plasma required for "sleep" in 50% of patients, and it is the concentration that induces a 50% halothane minimum alveolar concentration reduction in rats. A more than fourfold greater concentration of 32 μg/ml (133 μM total, 21 μM free drug), however, was required to achieve a 90% reduction in minimum alveolar concentration.[33] Regarding methohexital, a 3-mg/kg induction dose and a 0.05-mg $\cdot$ kg^{-1} $\cdot$ min^{-1} infusion yielded concentrations in plasma of 2.8 μg/ml (10 μM) in human volunteers,[49] which, considering the 73% plasma protein binding, represents a free concentration of $\approx$2.7 μM. Therefore, the lower concentrations of thiopental (10 μM) and methohexital (3 μM) used in this study, which represent routine clinical concentrations of free drug, were able to produce a decrease of 30% in the peak Ca^{2+} transient and a >40% reduction in glutamate release for thiopental. The greater potency of methohexital compared with thiopental in inhibiting the $[Ca^{2+}]_i$ transient peak and glutamate release appears to reflect the greater anesthetic potency of the former compound.

The ability of thiopental and methohexital to decrease intracellular Ca^{2+} transients and to decrease glutamate release provides a mechanism, through blocking excitatory synaptic transmission, by which they may induce a component of the anesthetic state. Such an action may be additive or synergistic, with the sedative/hypnotic actions mediated by the hyperpolarizing and inhibitory effects of $GABA_A$ receptor activation. In addition, decreased excitatory synaptic transmission and inhibition of entry of Ca^{2+} would be expected to decrease metabolic neuronal activity, which might explain the decreased oxygen consumption associated with administration of barbiturates.[50] Although these inhibitory actions on VGCC and glutamate release may contribute to the anesthetic state, they also may play a beneficial role in neurons subject to ischemic or hypoxic injury. Because glutamate release activates NMDA receptors and Ca^{2+} entry, a barbiturate-mediated decrease in its release, which also has been demonstrated in brain slices,[51] could be protective by preventing excitatory and potentially neurotoxic Ca^{2+} entry. In the presence of depletion of adenosine triphosphate, however, glutamate release from neurons may not be mediated by synaptic processes but may instead represent failure or reversal of processes of glutamate uptake.[52] Although inhibition of reversal of glutamate uptake has been demonstrated,[53] an additional important protective aspect of barbiturates may be the blockade of entry of Ca^{2+} through VGCC. Accumulation of Ca^{2+} through VGCCs,

by itself, also is protective by decreasing the entry of Ca^{2+}, which ultimately mediates neurotoxicity. Blockade of N-type VGCC by ω-conotoxin MVIIA (SNX-111) has been shown to be effective in decreasing neuronal loss and infarct size in models of global[54] or focal cerebral ischemia,[55] an effect also demonstrable for thiopental.[56] It is noteworthy that the protection afforded by SNX-111 was effective even when administered 24 h after the insult, suggesting that entry of Ca^{2+} *via* specific VGCCs may be important in mediating later events in the cascade that mediate neuronal death. In addition, blockade of P-type VGCC by ω-conotoxin MVIIC (SNX-230) decreased glutamate release but did not provide protection against the ischemic injury.[54] In models examining neural protection from ischemia, doses of thiopental and methohexital are used that are far higher than those used for anesthetic induction. Such doses are likely to achieve sustained concentrations in the intermediate range studied and thereby cause more profound reductions in Ca^{2+} entry.

Although barbiturate anesthetic agents have been found to have effects on a variety of ion channels, the barbiturate induction agents thiopental and methohexital at equipotent concentrations depressed VGCC-mediated Ca^{2+} entry into cultured CG neurons and the associated glutamate release. The direct depression of Ca^{2+} entry may contribute to their anesthetic action and their neuronal protective action.

References

1. Sieghart W: GABA$_A$ receptors: Ligand-gated Cl$^-$ channels modulated by multiple drug-binding sites. Trends Pharmacol Sci 1992; 13:446–50

2. Tanelian DL, Kosek P, Mody I, MacIver B: The role of the GABA$_A$ receptor/chloride channel complex in anesthesia. ANESTHESIOLOGY 1993; 78:757–76

3. Macdonald RL, Rogers CJ, Twyman RE: Barbiturate modulation of kinetic properties of the GABA$_A$ receptor channel of mouse spinal neurones in culture. J Physiol (Lond) 1989; 417:483–500

4. Macdonald RL, Skerritt JH, Werz MA: Barbiturate and benzodiazepine actions on mouse neurons in cell culture, Molecular and Cellular Mechanisms of Anesthetics. Edited by Roth SH, Miller KW. New York, Plenum, 1986, pp 17–25

5. Sevcik C: Differences between the actions of thiopental and pentobarbital in squid giant axons. J Pharmacol Exp Ther 1980; 214:657–63

6. Pancrazio JJ, Frazer MJ, Lynch C III: Barbiturate anesthetics depress the resting K$^+$ conductance of myocardium. J Pharmacol Exp Ther 1993; 265:358–65

7. Blaustein MP: Barbiturates block calcium uptake by stimulated and potassium-depolarized rat sympathetic ganglia. J Pharmacol Exp Ther 1976; 196:80–6

8. Heyer EJ, Macdonald RL: Barbiturate reduction of calcium-dependent action potentials: Correlation with anesthetic action. Brain Res 1982; 236:157–71

9. Gundersen CB, Umbach JA, Swartz BE: Barbiturates depress currents through human brain calcium channels studied in *Xenopus* oocytes. J Pharmacol Exp Ther 1988; 247:824–9

10. Ramón y Cajal S: La Textura del Sistema Nervioso del Hombre y los Vertebrados. Madrid, Moja, 1904

11. Novelli A, Reilly JA, Lysko PG, Henneberry RC: Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain Res 1988; 451:205–12

12. Pocock JM, Cousin MA, Nicholls DG: The calcium channel coupled to the exocytosis of L-glutamate from cerebellar granule cells is inhibited by the spider toxin, Aga-GI. Neuropharmacology 1993; 32:1185–94

13. Bossu JL, De Waard M, Fagni L, Stansfeld C, Tanzi F, Feltz A: Characteristics of Ca channels responsible for voltage-dependent Ca entry in rat cerebellar granule cells. Eur J Neurosci 1994; 6:335–44

14. Leighton C, Mathie A, Dolphin AC: Modulation by phosphorylation and neuro-transmitters of voltage-dependent calcium channel currents in cultured rat cerebellar granule neurons. J Physiol (Lond) 1994; 477:89P–90P

15. Randall A, Tsien RW: Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. J Neurosci 1995; 15:2995–3012

16. Pearson HA, Sutton KG, Scott RH, Dolphin AC: Characterization of Ca^{2+} channel currents in rat cerebellar granule neurones. J Physiol (Lond) 1995; 482:493–509

17. Huston E, Cullen G, Sweeney MI, Pearson H, Fazeli MS, Dolphin AC: Pertussis toxin treatment increases glutamate release and dihydropyridine binding sites in cultured rat cerebellar granule neurons. Neuroscience 1993; 52:787–98

18. Grignon S, Seagar MJ, Couraud F: ω-conotoxin sensitive calcium channels in cerebellar granule cells are not coupled to [^{3}H]glutamate release. Neurosci Lett 1993; 155:87–91

19. Travagli RA, Ulivi M, Wojcik WJ: γ-Aminobutyric acid-B receptors inhibit glutamate release from cerebellar granule cells: Consequences of inhibiting cyclic AMP formation and calcium influx. J Pharmacol Exp Ther 1991; 258:903–9

20. Graham ME, Burgoyne RD: Effects of Ca^{2+} channel antagonists on Ca^{2+} entry and glutamate release from cultured rat cerebellar granule cells. J Neurochem 1995; 65:2517–24

21. Zhao Z, Peng L: Glutamate effects on calcium homeostasis in cerebellar granule cells in primary cultures grown under depolarizing and nondepolarizing conditions. Synapse 1993; 13:315–21

22. Grynkiewicz G, Poenie M, Tsien R: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260:3440–50

23. Miao N, Frazer MJ, Lynch C III: Volatile anesthetics depress Ca^{2+} transients and glutamate release in isolated cerebral synaptosomes. ANESTHESIOLOGY 1995; 83:593–603

24. Clements JK, Lester RA, Tong G, Jahr CE, Westbrook GL: The time course of glutamate in the synaptic cleft. Science 1992; 258:1498–501

25. Kanai Y, Smith CP, Hediger MA: The elusive transporters with a high affinity for glutamate. Trends Neurosci 1993; 16:365–70

26. Regan LJ, Sah DWY, Bean BP: Ca^{2+} channels in rat central and

peripheral neurons: High-threshold current resistant to dihydropyridine blockers and ω-conotoxin. Neuron 1991; 6:269–80

27. Pearson HA, Sutton KG, Scott RH, Dolphin AC: Ca^{2+} currents in cerebellar granule neurones: Role of internal Mg^{2+} in altering characteristics and antagonist effects. Neuropharmacology 1993; 32:1171–83

28. Turner TJ, Adams ME, Dunlap K: Calcium channels coupled to glutamate release identified by omega-Aga-IVA. Science 1992; 258:310–3

29. De Waard M, Feltz A, Bossu JL: Properties of a high-voltage activated calcium current in rat cerebellar granule cells. Eur J Neurosci 1991; 3:771–7

30. Huston E, Scott RH, Dolphin AC: A comparison of the effect of calcium channel ligands and $GABA_B$ agonists and antagonists on transmitter release and somatic calcium channel currents in cultured neurons. Neuroscience 1990; 38:721–9

31. Labrakakis C, Muller T, Schmidt K, Kettenmann H: GABA(A) receptor activation triggers a Cl^- conductance increase and a K^+ channel blockade in cerebellar granule cells. Neuroscience 1997; 79:177–89

32. Nicoll RA, Eccles JC, Oshima T, Rubia F: Prolongation of hippocampal inhibitory postsynaptic potentials by barbiturates. Nature 1975; 258:625–7

33. Stone DJ, Moscicki JC, DiFazio CA: Thiopental reduces halothane MAC in rats. Anesth Analg 1992; 74:542–6

34. Gross RA, Macdonald RL: Differential actions of pentobarbitone on calcium current components of mouse sensory neurones in culture. J Physiol (Lond) 1988; 405:187–203

35. ffrench-Mullen JM, Barker JL, Rogawski MA: Calcium current block by (−)-pentobarbital, phenobarbital, and CHEB but not (+)-pentobarbital in acutely isolated hippocampal CA1 neurons: Comparison with effects on GABA-activated Cl-current. J Neurosci 1993; 13:3211–21

36. Bleakman D, Jones MV, Harrison NL: The effects of four general anesthetics on intracellular $[Ca^{2+}]$ in cultured rat hippocampal neurons. Neuropharmacology 1995; 34:541–51

37. Tottene A, Moretti A, Pietrobon D: Functional diversity of P-type and R-type calcium channels in rat cerebellar neurons. J Neurosci 1996; 16:6353–63

38. Matthews G: Neurotransmitter release. Annu Rev Neurosci 1996; 19:219–33

39. Takahashi T, Momiyama A: Different types of calcium channels mediate central synaptic transmission. Nature 1993; 366:156–8

40. Wheeler DB, Randall A, Tsien RW: Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. Science 1994; 264:107–11

41. Augustine GJ, Charlton MP, Smith SJ: Calcium action in synaptic transmitter action. Annu Rev Neurosci 1987; 10:633–53

42. Mulkeen D, Anwyl R, Rowan M: The effects of external calcium on long-term potentiation in the rat hippocampal slice. Brain Res 1988; 447:234–8

43. Wu L-G, Saggau P: Pharmacological identification to two types of presynaptic voltage-dependent calcium channels at CA3-CA1 synapses of the hippocampus. J Neurosci 1994; 14:5613–22

44. Simpson PB, Nahorski SR, Challiss RAJ: Agonist-evoked Ca^{2+} mobilation from stores expressing inositol 1,4,5-trisphosphate receptors and ryanodine receptors in cerebellar granule neurones. J Neurochem 1996; 67:364–73

45. Komai H, Rusy BF: Effect of thiopental on Ca^{2+} release from sarcoplasmic reticulum in intact myocardium. ANESTHESIOLOGY 1994; 81:946–52

46. Mantz J, Varlet C, Lecharny J-B, Henzel D, Lenot P, Desmonts J-M: Effects of volatile anesthetics, thiopental, and ketamine on spontaneous and depolarization-evoked dopamine release from striatal synaptosomes in the rat. ANESTHESIOLOGY 1994; 80:352–63

47. Burch PG, Stanski DR: The role of metabolism and protein binding in thiopental anesthesia. ANESTHESIOLOGY 1983; 58:146–52

48. Becker KE Jr: Plasma levels of thiopental necessary for anesthesia. ANESTHESIOLOGY 1978; 49:192–6

49. Breimer DD: Pharmacokinetics of methohexitone following intravenous infusion in humans. Br J Anaesth 1976; 48:643–9

50. Michenfelder JD: The interdependency of cerebral functional and metabolic effects following massive doses of thiopental in the dog. ANESTHESIOLOGY 1974; 41:231–6

51. Bickler PE, Buck LT, Feiner JR: Volatile and intravenous anesthetics decrease glutamate release from cortical brain slices during anoxia. ANESTHESIOLOGY 1995; 83:1233–40

52. Szatkowski M, Attwell D: Triggering and execution of neuronal death in brain ischaemia: Two phases of glutamate release by different mechanisms. Trends Neurosci 1994; 17:359–65

53. Bickler PE, Buck LT, Hansen BM: Effects of isoflurane and hypothermia on glutamate receptor-mediated calcium influx in brain slices. ANESTHESIOLOGY 1994; 81:1461–9

54. Valentino K, Newcomb R, Gadbois T, Singh T, Bowersox S, Bitner S, Justice A, Yamashiro D, Hoffman BB, Ciaranello R, Miljanich G, Ramachandran J: A selective N-type calcium channel antagonist protects against neuronal loss after global cerebral ischemia. Proc Natl Acad Sci U S A 1993; 90:7894–7

55. Bowersox SS, Singh T, Luther RR: Selective blockade of N-type voltage-sensitive calcium channels protects against brain injury after transient focal cerebral ischemia in rats. Brain Res 1997; 747:343–7

56. Drummond JC, Cole DJ, Patel PM, Reynolds LW: Focal cerebral ischemia during anesthesia with etomidate, isoflurane, or thiopental: A comparison of the extent of cerebral injury. Neurosurgery 1995; 37:742–9