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Acetazolamide and Amiloride Inhibit Pentobarbital-induced Facilitation of Nocifensive Reflexes

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Background: **Neuronal excitation may result from stimulation of γ-aminobutyric acid A ($GABA_A$) receptors that prolong the channel opening, depolarizing the postsynaptic membrane. Drugs such as acetazolamide or amiloride can block GABA depolarization. Barbiturates facilitate nociceptive reflexes and also prolong the $GABA_A$ channel open-time. To evaluate the possible mechanism, the authors studied the impact of acetazolamide and amiloride on pentobarbital-induced nocifensive reflex facilitation. Because nitric oxide (NO) is a mediator of reflex facilitation, the authors evaluated the effects of NO synthase inhibition.**

Methods: **Nocifensive reflex thresholds were quantified with the hind paw withdrawal latency from radiant heat (HPW latency) in the rat. Nocifensive reflexes were facilitated with intraperitoneal injection of pentobarbital (30 mg/kg). The authors tested the roles of GABA-mediated depolarization and NO in reflex facilitation by pretreatment with acetazolamide and amiloride and inhibition of NO synthase with L-NAME and 7-NI, respectively. Sedative effects of pentobarbital were evaluated with the righting reflex, the response to vibrissal stimulation, and plasma drug concentrations.**

Results: **Pentobarbital decreased the hind paw withdrawal latency from 11.2 ± 1 to 8.3 ± 1 s ($P < 0.001$). Pretreatment with each of the four test drugs limited the reduction in reflex facilitation after pentobarbital to 1.3 s or less, similar to the reduction seen after saline injection, without altering sedation. L-NAME increased plasma pentobarbital concentrations by 10% without changing the concentration associated with return of responsiveness.**

Conclusions: **Pentobarbital-induced nocifensive reflex facilitation was inhibited by all four tested drugs without evidence of increased sedation. The results are consistent with a role for $GABA_A$ receptor–mediated depolarization in barbiturate-induced hyper-reflexia. (Key words: GABA depolarization; nitric oxide; suppression of inhibition.)**

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A LARGE body of evidence suggests that the (γ-aminobutyric acid A ($GABA_A$) receptor complex is an important cellular target for general anesthetics.[1,2] Conventionally viewed as an inhibitory neurotransmitter (through Cl^--mediated postsynaptic hyperpolarization), GABA may be either excitatory or inhibitory, depending on the frequency of stimulation.[3,4] Transient reduction of $GABA_A$ receptor–mediated inhibitory postsynaptic currents (IPSCs) is thought to play a role in regulating *N*-methyl-D-aspartate (NMDA) receptor–dependent mechanisms of synaptic plasticity.[3,5]

Recently, authors from two laboratories[3,4] proposed that an anion gradient shift contributes to the excitatory postsynaptic effects of high-frequency stimulation of GABAergic interneurons. According to the anion shift model,[3,4] GABA plays a dual role in the inhibition and facilitation of synaptic transmission (fig. 1A). High-frequency stimulation, by increasing GABA release, prolongs the open-time of the $GABA_A$ channel. This effect causes the $GABA_A$ reversal potential (E_{GABA}) to shift from E_{Cl} (hyperpolarized) toward E_{HCO_3} (depolarized).[3,4] The resulting depolarization can recruit previously inactive receptors, for example, by relieving the magnesium–voltage–dependent block of the NMDA receptor–mediated calcium channel. Extracelllular-to-intracellular diffusion of carbon dioxide and regeneration of HCO_3^- through the action of carbonic anhydrase maintain the electrochemical gradient for HCO_3^-. The excitatory effects of $GABA_A$ receptor stimulation can be blocked[3,4] by increasing the cytosolic hydrogen ion concentration $[H^+]_i$ with amiloride[6] or by interfering with the rehydration of carbon dioxide with acetazolamide[7] (fig. 1B).

We speculated that GABA depolarization, through the anionic gradient shift[3,4] (fig. 1B), is involved in the excitatory effects of general anesthetics observed *in vivo*. Our hypothesis is that in neural circuits in which inhibition plays an important modulatory role, low concentrations of general anesthetics enhance synaptic transmission by GABA depolarization. To test this hypothesis in the intact animal, we evaluated the effects of pentobarbital on nociceptive withdrawal (nocifensive[8]) reflex

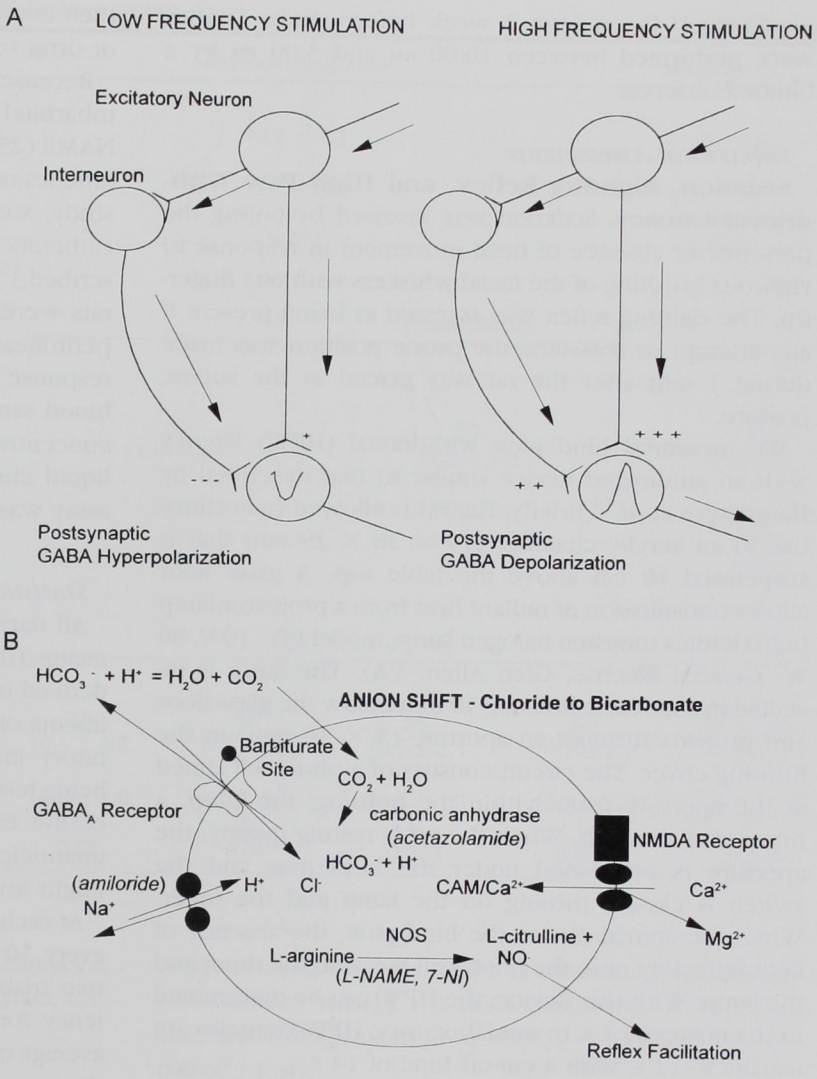

Fig. 1. Schematic representation of proposed mechanisms of pentobarbital-induced reflex facilitation. (*A*) In contrast to the inhibitory effects of GABA during low-frequency stimulation, depolarization-induced suppression of inhibition during high-frequency stimulation enhances synaptic transmission.[3,4] (*B*) The anion gradient shift mechanism for $GABA_A$ receptor–mediated depolarization.[3,4] Prolongation of the open-time of the $GABA_A$ receptor–chloride channel by pentobarbital[2,11] partially depolarizes the postsynaptic membrane, enhancing conductance at the NMDA receptor–mediated calcium channel. Increased intracellular calcium ion concentration $[Ca^{2+}]_i$ stimulates nitric oxide (NO) synthase, leading to increased synthesis of NO and reflex facilitation.[10] The anion gradient shift model[3,4] predicts that reflex facilitation by pentobarbital should be sensitive to drugs that increase cytosolic hydrogen ion concentration $[H^+]_i$ (amiloride) or impair rehydration of carbon dioxide by inhibiting carbonic anhydrase (CA) (acetazolamide). Nocifensive hyper-reflexia may also be mediated by NO and therefore sensitive to inhibition of NO synthase (L-NAME, 7-NI).

thresholds. Nocifensive reflexes are under segmental modulatory control by $GABA_A$ receptor–mediated mechanisms.[9] Facilitation of nocifensive reflexes occurs during NMDA receptor–, nitric oxide(NO)–mediated hyperalgesia (fig. 1B).[10] We chose pentobarbital because it is known to prolong the $GABA_A$ receptor channel open-time[2,11] and facilitates nocifensive reflexes.[12,13] If this hypothesized mechanism is correct (fig. 1B), pentobarbital-induced nocifensive reflex facilitation should be sensitive to acetazolamide and amiloride.[3,4] In addition, Staley *et al.*[3] predict that block of GABA-mediated reflex enhancement should occur without influencing GABA inhibitory effects. To test this prediction, we evaluated the effects of amiloride and acetazolamide on the suppression of two non-nociceptive reflexes by pentobarbital. The reflexes tested were head movement in response to brushing the facial whiskers and the righting response. Finally, we speculated that the facilitation of nocifensive reflexes by pentobarbital reflects activation of a normal physiologic mechanism for reflex enhancement. Because NO is involved in nocifensive reflex enhancement,[10] we evaluated the effects of NO synthase inhibition on pentobarbital-induced reflex facilitation.

Materials and Methods

The study protocols were approved by the Faculty of Medicine Animal Care Committee and were designed to comply with the guidelines of the Canadian Council for Animal Care and the International Association for the Study of Pain. Male Sprague Dawley rats (250–300 g) were housed in the medical vivarium (lights on: 7:00

AM–6:00 PM) for at least 1 week before study. Studies were performed between 10:00 AM and 3:00 PM by a blinded observer.

Behavioral Assessments

Sedation, Righting Reflex, and Hind Paw Withdrawal Latency. Sedation was assessed by noting the presence or absence of head movement in response to vigorous brushing of the facial whiskers with one fingertip. The righting reflex was assessed as being present if any attempt to reassume the prone position was made during 1 min after the rat was placed in the supine posture.

We measured hind paw withdrawal (HPW) latency with an automated device similar to that described by Hargreaves *et al.*[14] Briefly, the rat is allowed to acclimatize to an acrylic chamber (19 × 28 × 29 cm) that is suspended 10 cm above the table top. A glass floor allows transmission of radiant heat from a projector lamp bulb (Radius tungsten halogen lamp, model EJY, 19 V, 80 W; General Electric, Glen Allen, VA). The lamp is installed in a movable housing 40 mm below the glass floor and projects through an aperture (5 × 10 mm) in the housing cover. The circuit consists of a photocell aimed at the aperture from within the housing, the lamp, a timer, and a switch. When the rat is resting quietly, the aperture is positioned under the hind paw and the switch is closed, turning on the lamp and the timer. When the animal moves the hind paw, the absence of light reflecting onto the photocell turns off the timer and the lamp. With this device, the HPW can be determined to the nearest 0.1 s. In our laboratory, HPW latencies are usually 9–12 s, with a cut-off time of 14 s.

Experimental Protocol, Materials, and Drug Analysis

The effects of inhibition of carbonic anhydrase, and Na^+–H^+ exchange on reflex facilitation were tested by intraperitoneal injection of acetazolamide, 10, 50, or 100 mg/kg,[7] or amiloride, 100 mg/kg,[6] respectively. The influence of NO synthase inhibition was examined by the intraperitoneal injection of N^G-nitro-L-arginine-methyl ester (L-NAME; 2.5, 10, 25, or 50 mg/kg)[15] and the sodium salt of 7-nitro indazole (7-NI; 5 or 10 mg/kg),[16] 15 min (L-NAME), or 30 min (7-NI) before the pentobarbital injection. The vehicles for acetazolamide and amiloride were sodium bicarbonate (300 mM) and mannitol (1 M), respectively; L-NAME and 7-NI were dissolved in normal saline. The decrement in nocifensive reflex threshold during 180 min after the pentobarbital injection was then compared between animals pretreated with saline or drug vehicle and those treated with the study drugs.

Because L-NAME enhanced the sedative effects of pentobarbital (see Results), we next evaluated whether L-NAME (25 mg/kg intraperitoneal) altered the pharmacokinetics or pharmacodynamics of pentobarbital. For this study, we used rats instrumented with femoral artery catheters and partially restrained, as previously described.[13] Fifteen minutes after the injection of L-NAME, rats were injected with pentobarbital (30 mg/kg intraperitoneal). Every 10 min for eight measurements, the response to whisker stimulation was evaluated, and a blood sample (250 l) was drawn. Plasma pentobarbital concentrations were measured with high-performance liquid chromatography[13]; the limit of detection of the assay was $1 \cdot ml^{-1}$, and the accuracy was ±4%.

Statistical Analysis

All data are reported as the mean value ± SD of the mean. The number of animals in these experiments was derived from a power analysis based on previous experiments of reflex facilitation in our laboratory. The laboratory investigator was blinded to the drug and dose being tested. A second investigator monitored the results of the experiments for errors and to ensure that no unanticipated confounding factors were present that might invalidate the protocols.

At each measurement time (before and at approximately every 10 min after pentobarbital injection for 180 min), two trials were conducted in each animal. The HPW latency for that measurement time was calculated as the average of the two trials. To pool the data among animals in the same treatment group at each measurement time, the HPW values were normalized to the percentage of the control (postdrug, prepentobarbital) latency.[17] The normalized HPW curves for the treatment groups during the postinjection period were compared by one-way repeated measures analysis of variance (ANOVA).[18] The Student-Newman-Keuls test for multiple comparisons was applied *post hoc* to isolate significant ($P < 0.05$) individual group differences. Duration of loss of responsiveness to innocuous stimulation was analyzed by one-way ANOVA on ranks. The mean plasma pentobarbital time curve for animals injected with L-NAME was compared with the curve from saline-injected controls by one-way ANOVA of the mean values at each measurement time. The plasma concentration of pentobarbital associated with return of responsiveness to whisker stimulation was compared to the value from saline-injected controls with an unpaired Student *t*

Table 1. Effects of Study Drugs and Vehicles on HPW Latency (HPWL, s)

	Saline	Bicarbonate, 300 mM	Mannitol 1 M
Vehicles			
HPWL, control	10.5 ± 1.0	10.9 ± 1.3	11.2 ± 0.8
HPWL, postdrug	11.2 ± 1.3	11.1 ± 0.5	11.1 ± 1.2
n	7	7	6
P	0.320	0.732	0.869

	Amiloride	Acetazolamide		
Anion shift blockers				
Dose mg.kg^{-1}	100	10	50	100
HPWL, control	12.6 ± 1.2	11.9 ± 0.8	11.2 ± 1.3	11.5 ± 1.7
HPWL, postdrug	10.8 ± 1.3	11.9 ± 1.5	11.0 ± 1.2	11.1 ± 1.0
n	5	6	6	6
P	0.042	1.0	0.787	0.630

	7-Nitroindazole		L-NAME				
NOS inhibitors							
Dose mg.kg^{-1}	5	50	2.5	10	25	50	25 + L-Arg
HPWL, control	12.0 ± 1.6	11.7 ± 1.1	10.1 ± 0.6	11.3 ± 0.9	10.5 ± 2.1	10.7 ± 2.1	11.4 ± 2.1
HPWL, postdrug	11.5 ± 1.3	11.6 ± 1.5	10.8 ± 0.6	9.9 ± 0.6	10.6 ± 1.3	10.3 ± 1.0	11.5 ± 0.7
n	6	6	6	6	6	6	6
P	0.902	0.566	0.07	0.01	0.923	0.682	0.914

test. Analysis and graphics were performed with SigmaStat and SigmaPlot programs (Jandel Scientific, San Rafael, CA.

Results

Within 2 min after the pentobarbital injection, rats became unresponsive to vibrissal stimulation and lost the righting reflex. During the 180-min study after pentobarbital injection, HPW latency was decreased from by 26 ± 8% of control values (11.2 ± 1.1 to 8.3 ± 1.0 s), compared with a 4 ± 8% reduction in the saline-injected animals (10.6 ± 0.9 to 10.1 ± 0.8 s) ($t = 8.38$, $P < 0.001$, two-tailed Student t test). Responsiveness to vibrissal stimulation and the righting reflex returned 30–50 min after the pentobarbital injection.

Effects of Amiloride, Acetazolamide, and L-NAME on HPW Latency

There were no consistent changes in the baseline (pre-pentobarbital) HPW latency after injection of the study drugs or drug vehicles (table 1). Overall, in all animals, HPW latency was 11.1 ± 0.2 s before and 11.0 ± 0.1 s after study drug injection (n = 85, $t = 0.694$, $P = 0.489$, paired t test).

In animals treated with amiloride, acetazolamide, L-NAME, and 7-NI, the nocifensive reflex facilitation observed after pentobarbital injection was reduced (figs. 2 and 3). Inhibition of NO synthase blocked the pentobarbital-induced reduction in HPW latency in a dose-dependent fashion (fig. 3), an effect that was prevented by the coadministration of L-arginine with L-NAME. In the amiloride-treated animals there was an unexpectedly high death rate (3 of 11) 24 h after the experiments. Postmortem examination determined that these animals died of intestinal perforation with peritonitis. Because a significant block of HPW facilitation was detected with the 100-mg/kg dose after five animals, the amiloride study was discontinued at that point without proceeding to a dose-response study, as was done with acetazolamide.

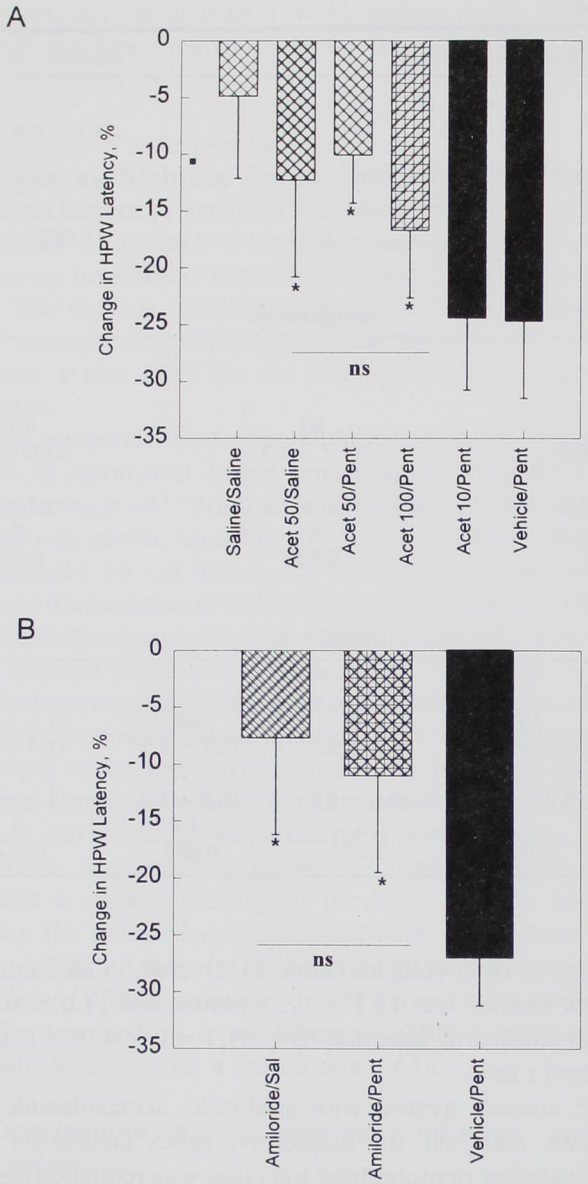

Fig. 2. Sensitivity of nocifensive reflex facilitation to drugs that interfere with the $GABA_A$ receptor–mediated anionic gradient shift. The figures show the mean change ± SD in hind paw withdrawal latency during 180 min after pentobarbital injection. Nocifensive reflex enhancement by pentobarbital was blocked by (*A*) Acetazolamide, 50 and 100 mg/kg (six rats per group) ($F = 17.69$, $P < 0.001$, *$P < 0.05$ *vs.* vehicle/pentobarbital and saline/saline) and (*B*) amiloride, 100 mg/kg (five rats in amiloride-treated animals, six animals in the vehicle/pentobarbital group) ($F = 32.78$, $P < 0.001$), *$P < 0.05$ *vs* vehicle/pentobarbital). ns = not significantly different.

Effects of Amiloride, Acetazolamide, 7-NI, and L-NAME on Responsiveness to Vibrissal Stimulation and the Righting Reflex

Treatment with amiloride and acetazolamide and 7-NI did not alter the sedative effects of pentobarbital (table 2), as measured by the response to whisker stimulation and the righting reflex.

The sedative effects of pentobarbital were enhanced by L-NAME in doses of 25 mg/kg or greater. In rats that received saline, or L-NAME in doses of 2.5 and 10 mg/kg, the righting reflex and responsiveness to vibrissal stimulation returned after 44 ± 7, 40 ± 7, and 45 ± 10 min compared to mean values of 57 ± 5 and 58 ± 10 min in the animals that received 25 and 50 mg/kg L-NAME ($F = 5.5$, $P = 0.001$, one-way ANOVA).

The plasma pentobarbital concentration associated with the return of responsiveness to vibrissal stimulation was similar in animals pretreated with intraperitoneal L-NAME, 25 mg/kg (n = 5, 10 ± 2 μg/ml, 39 ± 7 μM) compared to saline-pretreated controls (n = 9, 11 ± 1 μg/ml, 44 ± 3 μM) ($t = 1.2$, $P = 0.244$). L-NAME altered the pharmacokinetic profile of pentobarbital (fig. 4). Peak pentobarbital concentrations achieved in the two groups were similar (L-NAME [n = 5], 23 ± 1 μg/ml [93 ± 4 μM]; saline [n = 9], 21 ±

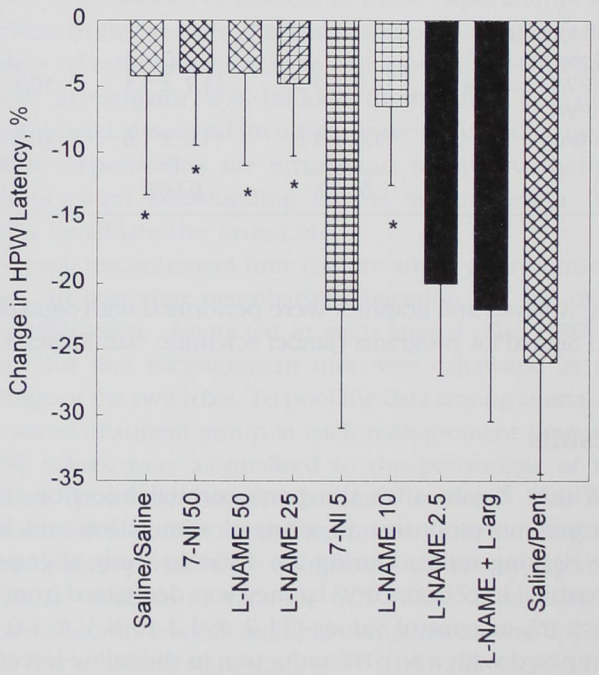

Fig. 3. Reduction in hind paw withdrawal latency for 180 min (mean values ± SD, six animals per group) after pentobarbital injection for L-NAME– and 7-NI–treated groups (n = 6 animals per group). L-NAME ($F = 27.7$, $P < 0.001$, one-way analysis of variance) and 7-NI ($F = 25.3$, $P < 0.001$) administered before pentobarbital blocked nocifensive reflex facilitation in a dose-dependent manner. The saline/saline group received no pentobarbital. All other groups were injected (intraperitoneally) with saline (saline/pentobarbital), L-NAME (2.5, 10, 25, 50 mg/kg) or L-NAME, 25 mg/kg, + L-arginine, 300 mg/kg) or 7-NI (5 or 50 mg/kg) 30 min before the pentobarbital injection. *Significantly different from saline/pentobarbital controls ($P < 0.05$).

Table 2. Duration of Unresponsiveness after Pentobarbital Injection

Study Drug or Vehicle (V)	n	Median Duration (min) of Loss of Responsiveness (25–75 percentiles)	
		Vibrissal Stimulation	Righting Reflex
Amiloride 100 mg · kg^{-1}	5	30 (30–40)	30 (30–40)
Mannitol (V)	6	30 (30–40)	30 (30–40)
Acetazolamide 10 mg · kg^{-1}	6	40 (30–50)	40 (30–50)
Acetazolamide 50 mg · kg^{-1}	6	40 (30–50)	40 (30–50)
Acetazolamide 100 mg · kg^{-1}	6	40 (30–50)	40 (30–50)
7 Nitroindazole 5 mg · kg^{-1}	6	35 (30–40)	35 (30–40)
7 Nitroindazole 50 mg · kg^{-1}	6	40 (30–40)	40 (30–40)
L-NAME 2.5 mg · kg^{-1}	6	40 (30–40)	40 (30–40)
L-NAME 10 mg · kg^{-1}	6	45 (40–50)	45 (40–50)
L-NAME 25 mg · kg^{-1}	6	60 (50–70)*	60 (50–70)*
L-NAME 50 mg · kg^{-1}	6	60 (50–70)*	60 (50–70)*
Saline (V)	7	45 (40–50)	45 (45–50)
Bicarbonate (V)	7	40 (30–50)	40 (30–50)

n refers to the number of animals.

* $P < 0.05$ *versus* saline, and L-NAME, 2.5 and 10 mg.kg^{-1}.

2 μg/ml [84 ± 8 μM], $t = 0.959$, $P = 0.357$). During the subsequent measurement times, plasma pentobarbital concentrations were increased in the L-NAME group ($F = 17.17$, $P = 0.003$, one-way ANOVA). The increase in plasma pentobarbital concentrations was consistent with the prolongation of loss of righting reflex seen in the unrestrained animals (table 2, fig. 4).

Discussion

These findings provide evidence for $GABA_A$ receptor-mediated excitation by pentobarbital in nocifensive reflex enhancement. Anesthetics alter information transfer in the nervous system. This modification in signaling may result in a change in behavior. The question that underlies the current study is, "How do anesthetics, at a molecular level, contribute to behavioral change, in this case enhanced nocifensive reflexes?" To relate these two requires an approach that begins from the behavioral change, and then proceeds to identify its central nervous system substrates. The current study represents a first step in this endeavor, by providing a link between mechanisms of $GABA_A$ receptor-mediated neuronal excitation described *in vitro*, with nocifensive reflex enhancement by pentobarbital *in vivo*. However, there are numerous interactions between the signaling and second-messenger pathways that are beyond the scope of the current study that could provide alternate explanations for our results. Investigation of these possibilities with *in vitro* methodologies, such as the spinal cord slice preparation, may constitute a reasonable next step.

In the current study, analgesic and sedative effects of the test drugs would represent significant confounding factors. We attempted to eliminate these with an appropriate study design. Can our results be explained by analgesic effects of amiloride, acetazolamide, L-NAME, or 7-NI? This appears to be unlikely because, after injection of these drugs, there were no changes in HPW latency that were consistent with analgesia.

Acetazolamide (50 mg/kg) administered without pentobarbital reduced HPW threshold more (−12 ± 9%) than saline (−5 ± 7%) ($P < 0.05$) (fig. 2), suggesting that acetazolamide may facilitate nocifensive reflexes. This may account for the lack of concentration dependence for the 50- and 100-mg/kg doses of acetazolamide.

Was the reduction in reflex facilitation caused by enhancement of sedative effects of pentobarbital? This also appears to be unlikely because amiloride, acetazolamide, and 7-NI did not alter the duration of loss of the righting reflex or the responsiveness to vibrissal stimulation caused by pentobarbital (table 2). L-NAME, in doses of 25 mg/kg or greater, enhanced the sedative effects of pentobarbital, delaying the return of responsiveness to vibrissal stimulation and the righting reflex. This en-

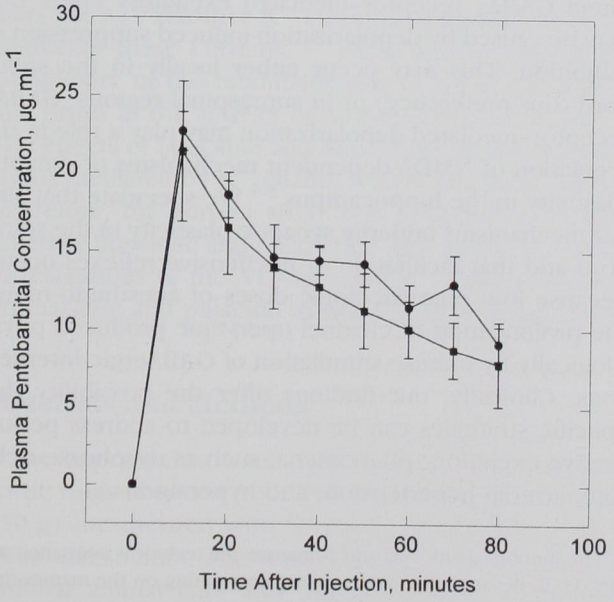

Fig. 4. L-NAME, 25 mg/kg, injected intraperitoneally 15 min before pentobarbital administration (circles, ± SD) increased mean plasma pentobarbital concentrations in comparison to the saline-injected animals (squares) during the first hour after injection ($F = 17.17$, $P = 0.003$).

hancement of sedation by the higher doses of L-NAME was consistent with the altered pharmacokinetics of pentobarbital by L-NAME (fig. 4).

Our findings are consistent with previous *in vitro* observations[3] that acetazolamide and amiloride block GABA-mediated excitation (nocifensive reflex enhancement) without altering GABA-mediated inhibition (possibly pentobarbital's sedative effects). These results suggest that the excitatory effects of pentobarbital are circuit specific, a finding that was previously been reported from *in vitro* studies in the hippocampus.[19]

Although NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are potentially important intermediaries in nocifensive reflex facilitation;[10] and investigation of the roles of these receptors in the current experimental model is confounded by the general anesthetic properties of specific antagonists for these receptors.[20,21]

In the spinal cord, pentobarbital can change the response profiles of some low-threshold neurons to resemble wide-dynamic-range neurons.[22] This is a potential mechanism for the reductions in HPW latency seen in the current study. On the basis of a lack of effect of pentobarbital on spontaneous neuronal activity, Collins *et al.*[22] argued that the unmasking of wide-dynamic-range neurons was caused by disinhibition or disruption of descending tonic inhibition. Our findings support a direct $GABA_A$ receptor-mediated excitatory effect that may be caused by depolarization-induced suppression of inhibition. This may occur either locally in the spinal cord (our preference) or in supraspinal regions. $GABA_A$ receptor-mediated depolarization may play a role in the regulation of NMDA-dependent mechanisms of synaptic plasticity in the hippocampus.[3,4] We speculate that similar mechanisms underlie synaptic plasticity in the spinal cord and that facilitation of nocifensive reflexes occurs because low pharmacologic doses of anesthetic mimic the prolongation of channel open-time produced physiologically by intense stimulation of GABAergic interneurons. Clinically, our findings offer the possibility that specific strategies can be developed to address perioperative excitatory phenomena, such as dysphoria, delirium, arterial hypertension, and hyperalgesia.

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