Preemptive Analgesic Effects of Steroid Anesthesia with Alphaxalone in the Rat Formalin Test

Evidence for Differential GABA_A Receptor Modulation in Persistent Nociception

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Background: The role of preemptive treatment with volatile and intravenous anesthetics has been examined in previous studies using the rat formalin test. Evidence describing analgesic properties of the gamma-amino butyric acid-ergic (GABAergic) steroid anesthetics, such as alphaxalone, suggest that they may suppress the development of central sensitization to pain. This study examined the preemptive effects of alphaxalone in comparison with other GABAergic anesthetics, propofol and pentobarbital.

Methods: The pain behavior of rats was evaluated (using the previously validated weighted scores method of behavioral rating) 15–60 min after subcutaneous hind paw injection of 50 μl 1.5% formalin. In each trial, anesthetics and their respective vehicles were administered by tail-vein injection either 0.5–10 min before or 5 min after, formalin injection. When analgesic effects were observed with any of these agents, further studies were conducted with a GABA_A receptor antagonist in an attempt to confirm a specific receptor-mediated action of the agent.

Results: Alphaxalone pretreatment produced transient analgesia in the early part of phase 2, which was not observed in rats posttreated with alphaxalone. The analgesic effect of alphaxalone was antagonized by picrotoxin, as well. Neither pentobarbital nor propofol showed any analgesic effects at the doses used in our study.

Conclusions: Whereas alphaxalone was shown to produce preemptive analgesia through its action at the GABA_A receptor, pentobarbital and propofol, which also are known to act at this site, showed no analgesic effects. The diversity of receptor subtypes and functional complexity of GABA_A receptors is such that steroid anesthetics may have effects that are different from other GABAergic agents. Further research into the role of progesterone metabolites and steroid anesthetics in the prevention of central sensitization may have clinical implications for the treatment of acute or chronic pain. (Key words: Agonists; gamma-amino butyric acid. Analgesics: alphaxalone. Anesthetics, steroid: alphaxalone. Pain: acute; chronic. Spinal cord: nociception.)

DURING the past 10 yr, basic and clinical research has suggested that noxious peripheral stimulation produces central nervous system sensitization, which subsequently influences pathologic pain processes. The formalin test has been used in a number of animal species as an experimental model of central sensitization to pain. In this test, subcutaneous injection of dilute formalin produces a biphasic nociceptive response with an early phase of intense pain in the first few minutes, followed later by a tonic phase of moderate pain occurring about 20–60 min after formalin injection. This behavioral biphasic nociceptive response to formalin corresponds to an increase in activity of dorsal horn neurons. The term preemptive analgesia refers to analgesic treatments administered to preemt central nervous system sensitization induced by surgical injury, and this paradigm has been studied both clinically and in animal models such as the formalin test. More recently, basic and clinical studies have evaluated the preemptive effects of general anesthetics. Specifically, Goto et al., using the rat formalin test, demonstrated that pentobarbital, but not propofol, produced preemptive analgesia, thus raising the question of whether agents that share gamma-aminobutyric acid (GABA) agonist properties can exert differential effects.
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on nociceptive transmission. Progesterone metabolites and anesthetic steroids such as alphaxalone (the potent constituent of the anesthetic Althesin) are known to act at gamma-amino butyric acid A (GABA_A) receptor sites both in the brain and spinal cord. Given previous evidence demonstrating analgesic effects of anesthetic steroids, evaluation of their role in the development of central sensitization to noxious inputs would be of interest. Thus, the purpose of this investigation was to evaluate the preemptive effects of steroid anesthesia with alphaxalone in the rat formalin test. For purposes of comparison within our study, we elected to additionally evaluate the effects of other GABAergic anesthetics, pentobarbital and propofol.

Methods and Materials

Animals

The following experiments were carried out under protocols approved by the Institutional Animal Care Committee of the Clinical Research Institute of Montreal. Male Long Evans rats (weighing 250–350 g) were used in these studies. The rats were housed in groups of four, had free access to food and water, and were maintained on a 12-h light cycle.

Tail Vein Injection

Anesthetics, antagonists, and vehicles were administered in a volume of 1–2 ml/kg by tail vein injection for a period of 30–60 s. After immobilizing the awake rat in a cloth restrainer, a tail vein was cannulated with a 25-G butterfly infusion set (Venisystems, Abbott Laboratories, Montreal, Canada) primed with injectate. Intravenous cannulation, as noted by free backflow of blood into the tubing, was followed by syringe attachment and injectate administration. Successful injection was confirmed by again observing blood backflow on disconnecting the syringe after injection. Rats receiving an incomplete injection because of needle dislodgment were excluded from the study.

Formalin Test

Formalin-induced pain behaviors were measured in rats that received an injection of 50 µl 1.5% formalin into the planter surface of one hind paw with a 27-G needle. For nociceptive testing, each rat was placed in a 30 cm × 30 cm × 30 cm polyethylene box with a mirror below the floor at a 45° angle to allow unobstructed view of the paws. A pain score was determined using the weighted scores method of behavioral rating devised by Dubuisson and Dennis and validated in our laboratory recently. Scoring involved measuring the amount of time spent in each of four behavioral categories: 0 = the injected paw is not favored, 1 = the injected paw has little or no weight on it, 2 = the injected paw is elevated and not in contact with any surface, and 3 = the injected paw is licked, bitten, or shaken. A weighted average pain score, ranging from 0 to 3, was calculated by multiplying the time spent in each category by the category weight, summing these products, and then dividing by the total time in each 5-min block of time (i.e., 300 s). Because rats in this study were anesthetized during phase 1 of the formalin test, pain scoring was performed only during phase 2, that is from 15 to 60 min after formalin injection.

Anesthetic Agents and Receptor Antagonists

For the study of alphaxalone (Research Biochemicals, Natick, MA) preliminary experiments sought to determine a drug dose that would produce loss of righting reflex for at least 5 min (the approximate duration of phase 1) yet allow sufficient recovery for nociceptive scoring in phase 2. For dose-response experiments, half of this determined dose also was studied. Dose-response studies of pentobarbital (Abbott Laboratories) and propofol (Zeneca Pharma, Mississauga, Canada) have been published recently by Goto et al., so only a single dose was assessed for those agents. The doses of pentobarbital (20 mg/kg) and propofol (10 mg/kg) were selected as the largest possible ones that would allow sufficient recovery for behavioral testing 15 min after formalin injection. For antagonist studies described later, 0.25 mg/kg picrotoxin (Research Biochemicals) was used. Picrotoxin was used to antagonize GABA_A receptors and the 0.25-mg/kg dose was selected as the largest possible one that produced neither convulsant nor antinociceptive effects on its own in the formalin test. Vehicle control groups included: (1) 0.9% saline for comparison with the pentobarbital group, (2) 10% intravenous fat emulsion (Liposyn II 10%, Abbott Laboratories) for comparison with the propofol group, and (3) 20% Cremophor EL (Sigma, St. Louis, MO) in saline for comparison with the alphaxalone group. Tables 1 and 2 summarize the experimental groups studied and the specific treatment protocols.

Experimental Paradigms

Preinjury Versus Postinjury Anesthetic Administration. To evaluate the specific preemptive effect
of the agents studied, a preinjury group was compared to a postinjury group (fig. 1A). In the preinjury groups, rats received anesthetics 1 min (alphaxalone, propofol and vehicles) or 10 min (pentobarbital) before formalin injection, so as to be anesthetized during phase 1 of the formalin test. In the postinjury groups, anesthetics or vehicles were administered 5 min after formalin injection, to allow the rats to be awake during phase 1, fully anesthetized in the early part of phase 2, yet recover fully from anesthesia to allow nociceptive scoring to be initiated 15 min after formalin injection.

**Antagonist Studies.** Agents found to have analgesic effects in paradigm 1 subsequently were studied with a GABA<sub>A</sub> receptor antagonist to determine whether these effects were mediated by a specific action at the implicated receptor site. Thus, alphaxalone, which acts at the GABA<sub>A</sub> receptor, was examined together with the GABA<sub>A</sub> receptor antagonist picrotoxin. Initially, the antagonist was administered intravenously as a preinjury injection immediately after anesthetic administration (i.e., drug/antagonist/formalin group). If the antagonist attenuated the analgesic effect, comparison was made to another group that received the antagonist 5 min after formalin injection (i.e., drug/formalin/antagonist group). Thus, we assessed the effect of maintaining drug action during phase 1, yet reversing it early in phase 2, to further examine the specific effects of preemptive administration of anesthetic agents (fig. 1B). By antagonizing anesthetic action after phase 1, the preemptive action is maintained, but any lingering effects of the drug in phase 2 are eliminated. Control groups received the antagonist either immediately before formalin (vehicle/antagonist/formalin) or 5 min after formalin (vehicle/formalin/antagonist).

**Data Analysis**

Nociceptive scores throughout the testing sessions were analyzed using a two-way, repeated-measures analysis of variance. Any main effects of group were explored further using analysis of simple main effects. Post hoc tests, used after significant group by time interactions, were performed using Newman-Keuls multiple comparisons.

**Results**

**Alphaxalone**

Rats receiving 1.5 mg/kg alphaxalone demonstrated a loss of righting reflex that recovered by 3 min after drug injection. The administration of 0.75 mg/kg had a subhypnotic effect in all rats. Figure 2 shows nociceptive scores of rats treated with alphaxalone. Figure 2A illustrates the dose-response relationship of pain scores for pretreatments with vehicle, 0.75 or 1.5 mg/kg alphaxalone. A significant group by time interaction was observed among these groups (treatment group: F(2,16) = 1.32, P > 0.05; time period: F(8,128) = 3.69, P < 0.01; group by time interaction: F(16,128) = 1.82, P < 0.05). At 20 and 25 min, only the 1.5 mg/kg group was different from vehicle (P < 0.05, Newman-Keuls post hoc comparison). Figure 2B compares preinjury treatment with postinjury treatment of vehicle versus 1.5 mg/kg of alphaxalone. This analysis also showed a significant group by time interaction (treatment group: F(3,20) = 1.82, P > 0.05; time period: F(8,160) = 7.48, P < 0.01; Group by time interaction: F(24,160) = 2.83, P < 0.01). Alphaxalone pretreatment (PRE) was different from both vehicle PRE and alphaxalone post-treatment (POST) at 20 and 25 min but only different from alphaxalone POST at 30 min (P < 0.05, Newman-Keuls post hoc comparison). This preemptive effect was not observed at the smaller dose of 0.75 mg/kg (data not shown). Surprisingly,
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Fig. 1. The comparison of preinjury versus postinjury treatment (A). In the preinjury group, the drug is administered before formalin injection and is active during phase 1 of the formalin test. In the postinjury group, drug is administered at the end of phase 1. The paradigm for studies of receptor antagonism (B). To further establish the preemptive effect of the anesthetic, antagonist administration at the end of phase 1 (drug active during phase 1) is compared with administration with the drug before formalin injection (drug antagonized during phase 1). Shaded stripe depicts the approximate duration of action of the administered agents.

Fig. 2. Dose-response effects of alphaxalone (0.75 and 1.5 mg/kg) pretreatment in the formalin test (A). Preinjury treatment compared with postinjury treatment of vehicle versus 1.5 mg/kg alphaxalone (B). Vehicle and 1.5 mg/kg alphaxalone compared with the combined administration of alphaxalone and 0.25 mg/kg picotoxin (C). Preinjury versus postinjury 0.25 mg/kg picotoxin when administered with either vehicle or alphaxalone (D). (§ ALX 1.5 PRE was different from CREM PRE, * ALX 1.5 PRE was different from ALX 1.5 POST or ALX/PIC/for, — CREM POST was different from CREM PRE, P < 0.05, Newman-Keuls post hoc comparison.)

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scores in the postinjury vehicle group were significantly less than in the preinjury vehicle group 15–20 min after formalin injection. Figure 2C compares vehicle and 1.5 mg/kg alphaxalone with the combined administration of alphaxalone and 0.25 mg/kg picrotoxin. This analysis also showed a significant group by time interaction (treatment group: F(2,16) = 2.62, P > 0.05; time period: F(8,128) = 5.16, P < 0.01; group by time interaction: F(16,128) = 1.82, P < 0.05). Alphaxalone PRE was different from both vehicle PRE and alphaxalone/picrotoxin/formalin (ALX/PIC/for) at 20 and 25 min but only different from ALX/PIC/for at 30 min (P < 0.05, Newman-Keuls post hoc comparison). Thus, the administration of picrotoxin immediately after alphaxalone (ALX/PIC/for) completely reversed the analgesic effect of alphaxalone. Figure 2D compares preinjury versus postinjury 0.25 mg/kg picrotoxin when administered with either vehicle or alphaxalone. A significant effect of treatment group was observed between ALX/PIC/for and ALX/for/PIC (treatment group: F(3,18) = 3.34, P < 0.05; time period F(8,144) = 24.90, P < 0.01; group by time interaction: F(24,144) = 0.52, P > 0.05) such that an unopposed drug action during phase 1 resulted in significantly lower scores across the entire session as compared with when the antagonist was present throughout the agonist action.

Pentobarbital and Propofol

Rats receiving 20 mg/kg pentobarbital exhibited only a sub hypnotic effect. Preliminary experiments with 25 and 30 mg/kg produced loss of righting reflex; however, at these doses, rats remained anesthetized well into phase 2, which obscured behavioral testing, thus precluding the use of these doses in our study. The administration of 10 mg/kg propofol produced loss of righting reflex, which was recovered by 10 min after formalin injection. Figure 3 shows that for both of these agents no differences were observed among preinjury, postinjury, or control groups. In figure 3A, no significant effect of treatment group or interactions were observed (treatment group: F(3,19) = 1.79, P > 0.05; time period: F(8,152) = 14.97, P < 0.01; group by time interaction: F(24,152) = 1.57, P > 0.05). In figure 3B, no significant effect of treatment group was observed, but a group by time interaction was observed (treatment group: F(3,17) = 0.83, P > 0.05; time period: F(8,136) = 9.10, P < 0.01; group by time interaction: F(24,136) = 1.67, P < 0.05). The postinjury vehicle group for propofol (intravenous fat emulsion) demonstrated significantly lower scores than the preinjury vehicle group 15–20 min after formalin injection (P < 0.05, Newman-Keuls post hoc comparison).

Discussion

These data show that 1.5 mg/kg preinjury alphaxalone treatment produced significant analgesia in the early part of phase 2 of the formalin test that was not seen in control or postinjury-treated rats. However, this effect was not observed at a smaller dose of 0.75 mg/kg. Thus, this study provides behavioral evidence of dose-dependent preemptive analgesia with systemic administration of the steroid anesthetic alphaxalone in the rat formalin test. The reversal of this analgesic effect with concomitant systemic administration of picrotoxin suggests that the action of alphaxalone at the GABA\textsubscript{A}...
receptor mediates the preemptive effect observed. One might think that the analgesia observed is simply a lingering effect of alphaxalone that appears to dissipate at 30 min after formalin injection. However, this is unlikely, because no such analgesic effect was observed in posttreated rats. Furthermore, that antagonism of alphaxalone action with picotoxin, after phase 1, does not influence its analgesic effect further reinforces the suggestion that drug effect during the first phase (as opposed to a lingering drug effect) is necessary for providing analgesia during phase 2.

It is noteworthy that the preemptive analgesic effect of alphaxalone was observed only during the first 15 min of phase 2. If the transient nature of this preemptive analgesic effect is not due to the dissipation of alphaxalone action, why then was the analgesic effect of alphaxalone not observed throughout all of phase 2? It is likely that because control and posttreatment pain scores during the latter part of phase 2 were considerably lower than in the early part of phase 2, there is a smaller interval of potential difference between these and the pretreated subjects, and it is difficult to demonstrate significant differences at these time points. Thus, it may have been possible to demonstrate a longer duration of analgesia using a higher concentration of formalin, for which pain scores in the control group would remain elevated longer. However, it also is true that pain scores in the pretreatment group did not decline in the same manner as did the vehicle and posttreatment group, further suggesting that the preemptive analgesia was quite transient. It should be noted, in any case, that the transient nature of the preemptive effect parallels recent clinical observations, and together, while supporting the concepts of injury-induced neural plasticity and preemptive analgesia, they do bring into question the clinical relevance of using pretreatments with short-acting anesthetics or analgesics to alleviate persistent postoperative pain.

While barbiturates and anesthetic steroids both act on GABA<sub>A</sub> receptors, the diversity of receptor subtypes and functional complexity of these receptors is such that these two classes of agents are in fact quite different in their pharmacodynamic effects on the central nervous system. Several recent studies have demonstrated analgesic effects with steroid anesthetics. One study demonstrated analgesia after intracerebroventricular administration of 3α-Hydroxy-5α-Pregnan-20-one in mice, and another showed a decrease in dorsal horn cell activity after intravenous administration of Althesin in cats. While these results may suggest both spinal and supraspinal analgesic actions of steroid anesthetics, the decreased activity of dorsal horn cells observed after systemic Althesin administration may provide an electrophysiologic correlate of our behavioral evidence that alphaxalone suppresses a proposed spinal sensitization to noxious stimuli.

Unlike alphaxalone, pentobarbital and propofol, which also act at GABA<sub>A</sub> receptors, did not demonstrate any antinociceptive effects in our study. While Franklin and Abbott also found no analgesia after pentobarbital pretreatment in a model similar to ours, Goto et al. observed that pentobarbital, but not propofol, did produce preemptive analgesia. In comparison with the recent study by Goto et al., certain methodological differences do exist. Some minor differences include the species of rats used, slight differences in the timing of drug administration and the time period of phase 2 testing (10–75 min after formalin injection vs. 15–60 min in our study). One factor that may play a more important role in explaining these discrepant results is the method of behavioral testing used in each of the studies. Both our study and that of Franklin and Abbott evaluated pain by observing several aspects of behavior including flinching, licking, biting, and favoring the injected limb; other investigators, including Goto et al., have counted the number of flinches observed. While both of these behavioral methods have been validated and widely used, it is not certain whether they may necessarily provide similar results. This may be particularly relevant in the current comparison where rats are needed to recover fully from anesthesia for the weighted scores method, but may not have to be fully conscious to assess flinching. Also of interest, a recent study by O'Connor and Abram, which also evaluated the effect of anesthetic pretreatment in the rat formalin test, found that while thiopental had no effect, propofol did significantly reduce flinching behavior. Aside from the possibility of differing effects associated with the behavioral testing method used in the formalin test, there do appear to be discrepancies in the degree of antinociceptive effects produced by propofol or barbiturates in other nociceptive tests. Furthermore, recent in vitro evidence from Jewett et al. showing that propofol, pentobarbital, and thiopental depress spinal nociceptive transmission in the neonatal rat spinal cord, might indicate how these agents could prevent the development of central sensitization to nociceptive inputs. However, as recently discussed by O'Connor and Abram, inhibition of descending inhibitory pathways by these agents may offset the spinally mediated...
analgesic effects produced, and explain why they may not always be effective in behavioral studies.

Attempting to understand the observed differences between these anesthetic agents also warrants a discussion of their respective pharmacokinetic differences. Given the temporal resolution of the rat formalin test, a major challenge encountered in studies of preemptive analgesia is selecting the appropriate dose and timing of drug pretreatment such that the experimental agent is active during phase 1 and redistributed away from the central nervous system by the time phase 2 begins. Thus, the observed hypnotic effect is used to target the peak effect of drug action. With respect to their hypnotic effects at the doses studied, alfaxalone and propofol were comparable regarding to their onset and duration of action whereas pentobarbital exhibited a slower onset and longer duration of action. Another concern about drug distribution is that of differential drug access to the brain versus that of the spinal cord. While we suggested earlier that the differences observed between alfaxalone and pentobarbital/propofol may be related to pharmacodynamic diversity of action, an equally feasible explanation is that alfaxalone administration results in comparatively higher spinal cord tissue drug levels where it is proposed that the sensitization takes place.

It is noteworthy that in posttreated vehicle controls (cremophor and intralipid), pain scores at 20 min were significantly lower than those of the pretreated controls. Although a trend toward lower scores was also observed at this time in the posttreatment saline group, this was not statistically significant. We believe it is unlikely that this observed effect is due to some pharmacologic action of the vehicles themselves. A more feasible explanation is the possibility that pain scores recorded 15–20 min after formalin injection were reduced by stress-induced analgesia caused by restraint and tail vein cannulation of the subjects, which took place 5 min after formalin injection. That pain scores were low in the postformalin vehicle groups but not in the postformalin anesthetic groups may be explained by anesthetic inhibition of descending inhibitory pathways, which would likely block the pathways mediating stress-induced analgesia.

In conclusion, this study provides behavioral evidence for a possible role of anesthetic steroids such as alfaxalone in inhibiting the development of persistent nociceptive states through a selective action on GABAergic receptors. Our data do not demonstrate similar effects for pentobarbital or propofol, suggesting that the effect observed with alfaxalone possibly is mediated through action at different GABAergic receptor subtypes. Further basic and clinical research into the role of progestosterone metabolites and/or anesthetic steroids in the prevention of central sensitization to pain may have clinical implications for the management of acute or chronic pain.

References
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