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# Central XYLAZINE-Induced Antinociception, but not Peripheral, is Mediated by $Ca^{2+}$-Activated Cl- Channels (Caccs) in Rats

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## Abstract

Xylazine, an $\alpha_2$-adrenergic agonist traditionally used as a veterinary sedative, has been introduced into clinics to treat pain. Despite its aesthetic effects, some studies suggest that xylazine may interact with endogenous targets to promote analgesia, such as the cannabinoid and opioid systems or even through HCN channel hyperpolarization; therefore, this study aimed to evaluate the peripheral and central roles of $Ca^{2+}$-activated Cl- channels (CaCC) in xylazine-induced antinociception. Male Wistar rats were used to assess the nociceptive threshold with the mechanical paw pressure test. $PGE_2$ (2 µg) was injected into the right hind paw as a hyperalgesia stimulus. Niflumic acid, a selective CaCC blocker, was injected into the hind paw (32 µg/paw) or via the intrathecal route (2, 4, and 8 µg). Xylazine exhibited peak antinociceptive activity 5 minutes after intrathecal injection (10 µg) and also peripherally after hind paw injection (100 µg/paw). Niflumic acid reversed the xylazine-induced analgesic effect when injected into the spinal cord, but not in the hind paw. In conclusion, xylazine may induce activation of $Ca^{2+}$-activated Cl- channels as part of its spinal analgesic mechanism in an acute pain model.

**Keywords:** $Ca^{2+}$-Activated Cl- Channels; Niflumic Acid; Peripheral Antinociception; $PGE_2$ Acute Pain Model; Xylazine

**Abbreviations:** °C - celcius degrees; µl - microliter; AM - Ante Meridiem (before noon); CaCC - Ca2+-activated Cl- channels; DMSO - dimethyl sulfoxide; DRG - dorsal root ganglia; g - grams; HCN - Hyperpolarization-activated Cyclic Nucleotide-gated channels; Kg - Kilograms; L - liter; mg – miligrams; PGE2 - prostaglandin E2; PM - Post Meridiem (after noon); USA - United State of America; µg - micrograms; µmol - micromole

## Introduction

N-(2,6-dimethylphenyl)-5,6-dihydro-4H-1,3-thiazin-2-amine, commonly known as xylazine, is a drug first synthesized by Bayer in 1962, which initially promoted its use as an antihypertensive agent [1]. Later, its anesthetic properties were identified, leading to its widespread use in veterinary medicine as a sedative, antinociceptive, and muscle relaxant [2]. However, due to its significant hypotensive effects and central nervous system depression, it was not approved for human use [3]. It was first described that the effects of xylazine were mainly mediated through $\alpha_2$-adrenoceptor agonism

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[4]. Since then, several additional mechanisms of action have been reported. Specifically, in the field of analgesia, xylazine may induce antinociception through multiple pathways, including activation of postsynaptic $\alpha_2$-adrenoceptors [5], modulation of the endogenous opioid system [6], involvement of the endocannabinoid system [7], and inhibition of hyperpolarization-activated cyclic nucleotide-gated ion channel (HCN channel) currents [8].

Similar to HCN channels, $Ca^{2+}$-activated Cl- channels (CaCCs) are present in excitable cells like smooth muscle and various neurons, including those in the dorsal root ganglion (DRG), spinal cord, and autonomic system [9]. When these channels open, they cause membrane hyperpolarization, which depends on the chloride equilibrium potential and the resting membrane potential. These processes help produce an antinociceptive effect in afferent neurons through a hyperpolarization mechanism [10]. Based on the data presented, this study aims to determine whether peripheral and intrathecal injections of xylazine produce antinociception in an acute pain model induced by $PGE_2$ and if CaCCs are involved in this process.

## Material and Methods

### Animals

Male Wistar rats, weighing between 170 and 200 g and obtained from the Central Animal Facility of the Federal University of Minas Gerais in Minas Gerais, Brazil, were used in the experiments. The animals were housed in plastic cages with bedding made from forage shavings, with free access to water and food, and kept in the testing room for 2 days prior to the experiments for habituation. They were maintained in a temperature-controlled environment (24°C ± 2°C) with a 12-hour light/dark cycle (7:00 AM to 7:00 PM). All tests were conducted during the light phase (8:00 AM to 5:00 PM). All animal procedures and protocols were approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (protocol number 191/2023) and complied with the guidelines for assessing experimental pain in animals [11].

### Algesimetric method

Hyperalgesia was induced by injecting prostaglandin $E_2$ ($PGE_2$; 2 µg) subcutaneously into the plantar surface of the hind paw. The mechanical nociceptive threshold was measured by assessing the response to a paw pressure test, as described by Randall and Selitto (1957) [12]. An analgesimeter (Ugo-Basile, Varese, Italy) was used to apply a steadily increasing force to the rat's paw. The weight in grams (g) needed to trigger a paw withdrawal response was recorded as the nociceptive threshold. A cut-off value of 300 g was set to reduce the risk of paw injury. The nociceptive threshold, expressed in grams, was calculated as the average of three consecutive trials. The peak effect after $PGE_2$ injection was considered to occur at 180 minutes (the 3rd hour), at which point Xylazine was administered. For time-response experiments, measurements were taken at zero minutes and at 185, 190, 200, and 210 minutes. In delta (Δ) experiments, measurements were recorded at zero minutes and during the 3rd hour, with Δ calculated as the difference between these values. An n=4 was used for all experimental groups tested.

### Experimental design

In all experiments, $PGE_2$ was injected into the subcutaneous plantar surface of the right hind paw in a final volume of 100 µl. For intrathecal administration, rats were shaved in the dorsal lumbar region. After sedation with isoflurane (3.5 %) (CRISTÁLIA®, Brazil), xylazine and niflumic acid were injected in a volume of 20 µl using a 13 x 0,3 mm needle attached to a hypodermic syringe (BD®, Brazil) directly into the subarachnoid space between the sixth and seventh lumbar vertebrae [13]. This volume remains constant for the drugs and their respective vehicles. An animal group treated with 4 % lidocaine (20 µl) was used to confirm injection efficacy, showing temporary paralysis of the posterior limbs (data not shown). Prostaglandin $E_2$ ($PGE_2$, Sigma, USA) was dissolved in 2% ethanol. Xylazine (10% Syntec, BRA) was dissolved in physiological saline, and Niflumic acid (Sigma, USA) was dissolved in 10% DMSO in saline.

### Statistical analyses

All results were analyzed using GraphPad Prism 10.1 and are shown as mean ± SEM. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A difference was considered significant when $P < 0.01$.

## Results

When Xylazine was administered intrathecally (10 µg), it fully reversed $PGE_2$-induced hyperalgesia, bringing the nociceptive threshold back to baseline (Figure 1A). When injected into the hind paw, xylazine produced antinociception at a dose of 100 µg (Figure 1B). The maximum antinociceptive effect was observed 5 minutes after injection. Intrathecal administration of niflumic acid (2, 4, and 8 µg), a CaCC blocker, dose-dependently reduced xylazine-mediated antinociception (10 µg), with the 8-µg dose completely reversing the effect (Figure 1A). However, when injected into the hind paw, niflumic acid (32 µg/paw) did not reverse xylazine-induced analgesia (100 µg). Niflumic acid alone did not alter the nociceptive threshold in animals injected with $PGE_2$ or vehicle in both routes of administration (Figure 1).

## Discussion

Green, 1975 [14], described one of the earliest reports on xylazine analgesia, highlighting the challenges in measuring antinociception due to sedative effects following intramuscular injection. The author subjectively noted that a dose of 12 mg/Kg produced "good" analgesia, although it was insufficient and suitable only for superficial procedures in mice used for animal experiments. The subjectivity of Green's results underscores the importance of accurate and controlled methods of algesimetry in animal research. The development of tests such as the paw pressure test [12], tail flick test [15], and von Frey test has provided researchers with reliable tools to characterize central and peripheral algesimetry [16].

Using the paw pressure test, our group previously demonstrated the effectiveness of xylazine in producing analgesia after hind paw injection, administered 5 minutes before the peak action of

$PGE_2$, based on the dose and methodology used in this study [17]. In that research, the 100 µg dose completely eliminated pain responses when injected into the right hind paw of rats. Both the effect and the efficacy of the antinociceptive response against the $PGE_2$ model were confirmed, without systemic effects. Our group has also examined the peripheral mechanisms involved in that xylazine-induced analgesia. We have shown its reliance on the endocannabinoid system [18], endogenous opioid pathways [19], and the l-arginine/nitric oxide/cyclic GMP/KATP pathway [17, 20].

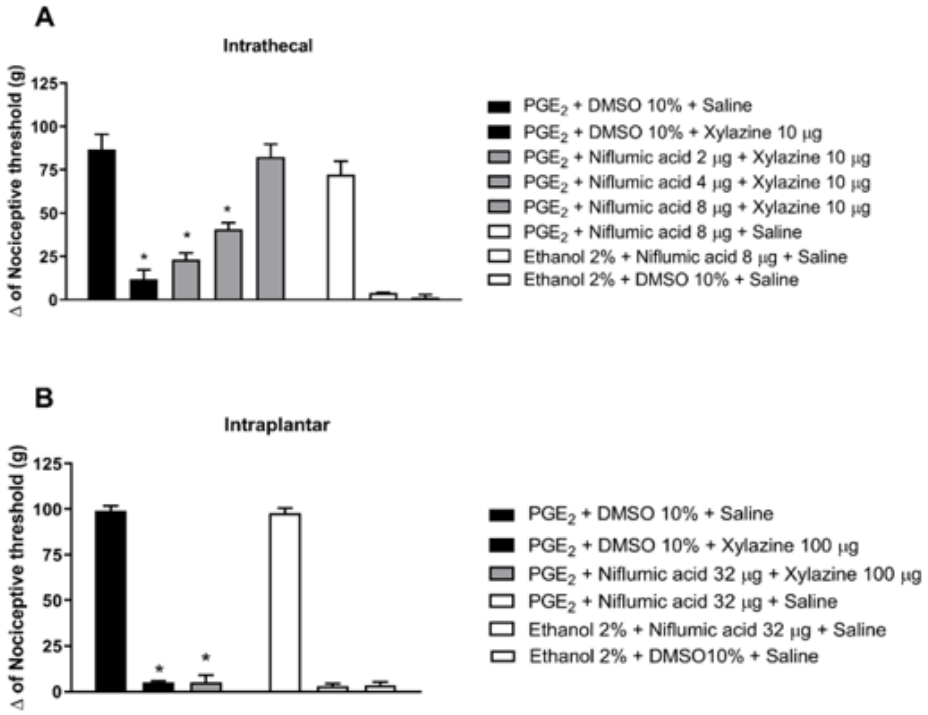


**Figure 1:** Effect of intrathecal (A) or Intraplantar (B) administration of $Ca^{2+}$-activated Cl- channels (CaCC) blocker (niflumic acid) on xylazine-induced antinociception: Niflumic acid was injected via intrathecal (2, 4 and 8 µg) or intraplantar (32 µg) 2h:45 min after 2 µg $PGE_2$ administration in the right hind paw. Xylazine was injected via intrathecal (10 µg) or intraplantar (100 µg) 5 minutes before the third hour of PGE2 injection according to the peak of hyperalgesia induced. * Indicates a significant difference from the ($PGE_2$ + Sal + DMSO 10%) injected group. # indicates a significant difference when compared with ($PGE_2$ + Xylazine + DMSO 10%). $p<0.01$, One-way ANOVA followed by the Bonferroni post-test.

The central administration of xylazine has also been shown in the literature to produce dose-dependent antinociceptive effects at doses of 5, 10, and 20 µg via the intracerebroventricular route [6]. Goodchild et al. (1996) [21] reported that, when injected intrathecally, xylazine depends on the adrenergic system for its antinociceptive effect; however, neither opioid nor Gabaergic propriospinal neurons are involved in mediating this effect. Several studies have shown that opening ATP-sensitive $K^+$ (KATP) channels is the final step in the peripheral antinociceptive mechanism of certain drugs, leading to neuronal membrane hyperpolarization [22-24]. However, hyperpolarization, which may block pain signal transmission, can also be mediated by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels or $Ca^{2+}$-activated Cl- (CaCC) channels [25].

It was demonstrated that thresholds for both mechanical and thermal nociceptive tests increased in a dose-dependent manner following xylazine administration (30 mg/kg and 40 mg/kg) in both HCN1+/+ and HCN1-/- mice [8]. However, in HCN1+/+ mice, these doses resulted in significantly higher thresholds compared to HCN1-/- mice. Whole-cell patch clamp recordings showed that xylazine inhibited HCN1 and HCN2 ion channel currents, causing a dose-dependent reduction of hyperpolarization-activated currents by xylazine (12.5-100 µmol/L). Similar to HCN channels, $Ca^{2+}$-activated Cl- channels (CaCCs) may also contribute to membrane hyperpolarization, and their role in the peripheral antinociceptive effects of opioids and cannabinoids has been reported [26, 27]. However, their part in xylazine-induced antinociception has not yet been studied.

In our study, intrathecal injection of niflumic acid (2, 4, and 8 µg), a selective CaCC blocker, dose-dependently reversed the analgesic effect of xylazine when given 5 minutes before its administration. In contrast, injecting niflumic acid (32 µg) into the hind paw did not affect xylazine-induced analgesia. This dose was the same as that which fully antagonized the peripheral antinociceptive effect of δ-opioid receptor activation [26]. These findings indicate that $Ca^{2+}$-activated Cl- channels contribute to the central, but not peripheral, part of xylazine-induced antinociception through a hyperpolarization-dependent mechanism.

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## Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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