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Research Article
Peripheral Antinociceptive Effects of *Parawixia bistriata* Venom and Glutamatergic Antagonists in the Formalin Test: Evidence of a Multi-Target Analgesic Mechanism

[1]Patrícia Frizo de Carvalho e Oliveira, [1]Marcus Vinicius Batista Celani, [2]Ana Paula Nascimento de Lima, [2]Alessandra Mussi Ribeiro, [3]Andreia Cristina Karklin Fontana and [1,4]Wagner Ferreira dos Santos

[1]Laboratory of Neurobiology and Venoms, Department of Biology, College of Philosophy, Sciences and Literature, University of São Paulo, Ribeirão Preto, São Paulo, Brazil
[2]Laboratory of Neuroscience and Bioprospecting of Natural Products Laboratory, Department of Biosciences, Federal University of São Paulo, Santos, Brazil
[3]Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, United States
[4]Institute of Neurosciences and Behavior, Ribeirão Preto, São Paulo, Brazil

Abstract

Background and Objective: Animal venoms are rich sources of compounds targeting nociceptive pathways. The venom of the spider *Parawixia bistriata* contains neuromodulatory compounds, but its analgesic potential is unknown. This study aimed to evaluate the peripheral antinociceptive effects of *Parawixia bistriata* venom and glutamatergic antagonists using the formalin test and to investigate the possible involvement of a multi-target analgesic mechanism in modulating nociceptive responses. **Materials and Methods:** Thirty-two adult male Wistar rats (≈215 g) were used under approved ethical guidelines (CEUA No. 06.1.91.53.4). Crude *Parawixia bistriata* venom was prepared and compared with MK-801 and CNQX in a formalin-induced nociception model. Treatments were administered via plantar injection using pre- and post-treatment protocols. Nociceptive behaviors were recorded and analyzed using non-parametric statistical tests ($p<0.05$). **Results:** Venom of *Parawixia bistriata* significantly reduced formalin-induced nociceptive behaviors in both phases of the test. In the prophylactic protocol, it decreased licking/lifting by 56.6% and paw withdrawal by 44.8% ($p<0.05$). MK-801 reduced only licking/lifting, while CNQX showed no effect. In the therapeutic protocol, venom and high-dose CNQX attenuated late-phase licking/lifting, with venom also reducing paw withdrawal. Overall, the venom showed broader and more consistent antinociceptive effects than selective glutamatergic antagonists. **Conclusion:** The venom of *P. bistriata* spider is a compelling source for developing novel peripheral analgesic strategies.

Key words: Pain modulation, formalin test, spider venom, glutamate transporters, GABA, peripheral sensitization

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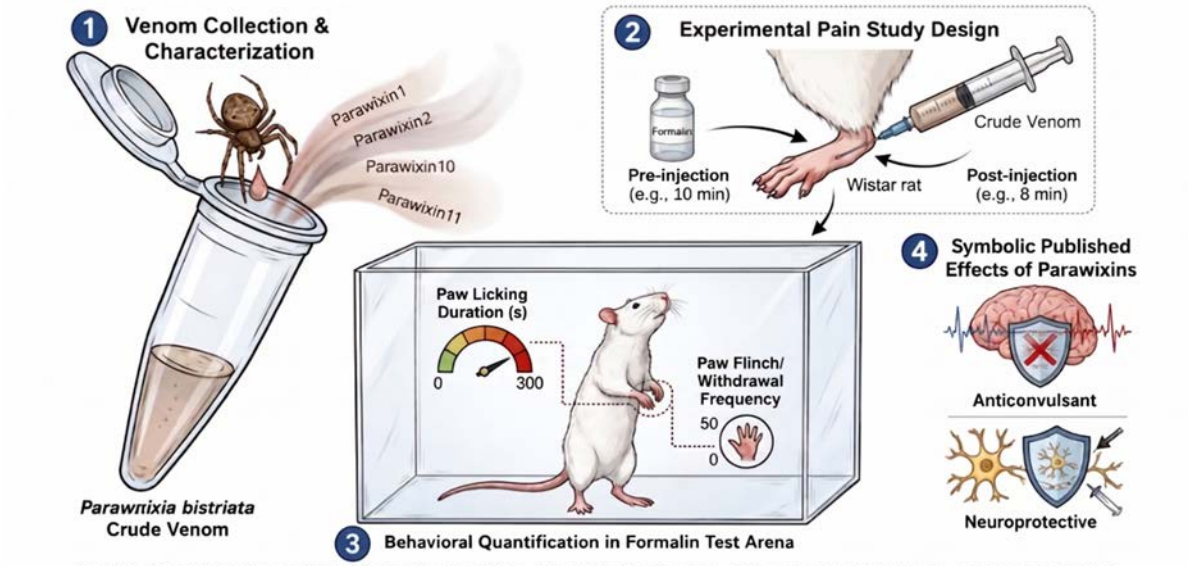
Corresponding Author: Wagner Ferreira dos Santos, Neurobiology and Venoms Laboratory, Department of Biology, College of Philosophy, Sciences and Literature, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

Graphical Abstract



Graphical abstract of the study. (1) Crude venom from *Parawiixia bistriata* was collected and characterized, revealing parawixins (Parawixin1, Parawixin2, Parawixin10, Parawixin11). **(2)** Experimental pain was induced in Wistar rats using the formalin test, with crude venom administration before formalin injection (pre-injection) and assessment during the post-injection phase. **(3)** Pain-related behaviors, including paw licking duration and paw flinch/withdrawal frequency, were quantified in a standardized arena. **(4)** Parawixins exhibit anticonvulsant and neuroprotective effects, highlighting their therapeutic potential.

INTRODUCTION

Pain, a crucial protective sensation, involves complex peripheral and central mechanisms. Nociceptive signals are initiated by the activation of Aδ and C fiber nociceptors and are modulated at the spinal level, as described by the Gate Control Theory[1]. Glutamatergic neurotransmission, mediated primarily through AMPA and NMDA receptors in the dorsal horn, is fundamental for both immediate nociceptive transmission and the central sensitization underlying chronic pain states[2,3], which involve functional and structural plasticity in the dorsal horn[4]. The formalin test effectively models this progression: Phase 1 (0-10 min) results from direct chemical activation of nociceptors, while Phase 2 (15-60 min) reflects ensuing inflammatory and central sensitization processes[3,5].

Current analgesic pharmacotherapy, including NSAIDs, opioids and anticonvulsants, is often limited by side effects, tolerance or inadequate efficacy[6,7]. Natural products have historically been invaluable, yielding drugs like morphine, aspirin and the venom-derived peptide ziconotide, an N-type calcium channel blocker for severe pain[8,9], highlighting the clinical value of toxins that target specific nociceptive pathways[10]. Spider venoms, in particular, are rich in peptides that modulate key ion channels (NaV, CaV, KV) involved in nociception[11,12], serving as tools for the development of new peptide-based analgesics[13].

The venom of the spider *Parawixia bistriata* is a source of neuroactive compounds that bidirectionally modulate synaptic transmission. Isolated acyl-polyamines from this venom include Pwx1, which enhances glutamate uptake and mitigates excitotoxicity[14,15] and Pwx2, an inhibitor of GABA and glycine uptake with anticonvulsant and neuroprotective properties[16,17]. Other components, such as Pwx10 and Pwx11, show neuroprotection against NMDA toxicity and anticonvulsant effects, respectively, further implicating glutamatergic pathway modulation[18,19]. Despite this well-characterized neuromodulatory profile, the analgesic potential of *P. bistriata* crude venom remains unexplored.

Given the central role of peripheral glutamatergic signaling in nociceptive transduction and sensitization, we employed a comparative pharmacological approach using the selective NMDA receptor antagonist MK-801 and the AMPA/kainate antagonist CNQX as positive controls. This allows us to discern whether the venom's effects are mediated primarily through localized glutamatergic blockade or arise from a more complex, multi-component interaction with the nociceptive system.

As the first study to investigate the analgesic potential, the use of crude venom allows for a broad initial screening. This approach is essential because the observed biological activity may arise from synergistic interactions between multiple components or from yet-unidentified molecules present in the whole venom. By demonstrating an analgesic

effect at this stage, the study establishes a foundation and rationale for future, more targeted efforts to isolate and characterize specific compounds responsible for the activity.

Therefore, this study investigated the effects of local peripheral administration of *P. bistriata* crude venom, compared to these glutamatergic antagonists, on formalin-induced nociceptive behaviors in rats during both acute and inflammatory phases[5].

MATERIALS AND METHODS

Animals and ethics: The experiments were conducted from January to May 2020. The animals were housed at the Biology Center of the USP- Ribeirão Preto (FFCLRP) and all behavioral tests were performed at the Laboratory of Neurobiology and Venoms, FFCLRP/USP. Thirty-two adult male Wistar rats (approximately 215 g body weight) were obtained from the central animal facility of the University of São Paulo. Animals were housed under standard controlled conditions (25°C, 12-hrs light/dark cycle) with ad libitum access to food and water. Before experimental procedures, all animals were acclimated to the test apparatus for 30 minutes daily over three consecutive days.

This study was conducted in strict accordance with the ethical guidelines for animal research established by the Brazilian Society for Neuroscience and Behavior (SBNeC), which were reinforced at the time of the experiments and incorporate the principles of the International Guiding Principles for Biomedical Research Involving Animals (CIOMS). All procedures were performed in compliance with Brazilian federal law governing the use of animals in scientific research (Law No. 11.794; CEUA protocol nr: 06.1.91.53.4). The experimental design and all techniques were rigorously planned to minimize animal suffering, with every effort made to reduce potential pain, discomfort or distress.

Venom and drugs: The *P. bistriata* spiders were collected in São Paulo State, Brazil. Crude venom was obtained from four pooled glands from two female spiders, homogenized in sterile saline and centrifuged to yield a stock solution of 20 mg/mL (protein concentration determined by the Lowry method)[20]. The concentrations used were based on pilot studies. Additionally, the following drugs were used: MK-801 (NMDA receptor antagonist, 1 µM, CNQX (AMPA/kainate receptor antagonist, 1 µM and 10 µM), 5% formalin and phosphate-buffered saline (PBS, pH 7.4, as control). All reagents were from Sigma-Aldrich (USA).

Behavioral protocols and analysis: Local injections (30 µL for drugs/PBS, 15 µL for venom or formalin) were administered into the plantar surface of the right hind paw using a 50-µL Hamilton syringe. Two protocols were employed: (1) Pre-treatment: Injection of drug, venom or PBS, followed 10 min later by formalin at the same site. (2) Post-treatment: Formalin injection at time 0, followed by drug, venom or PBS at 8 min. Behaviors were scored in 5-min intervals: The total duration (seconds) of licking/lifting of the injected paw (a complex, protective behavior) and the number of paw withdrawals (a brief reflexive lift). Data from minutes 5-10 post-injection were excluded to avoid confounding effects from the injection procedure itself, with time measured from the moment of formalin injection. Rat behavior was recorded in videotapes for subsequent observation.

Statistical analysis: Data are presented as Mean±Standard error of the mean (SEM). Inter-group comparisons across time points were analyzed using the Kruskal-Wallis test. Multiple comparisons across time intervals within groups were evaluated with the Friedman test. The significance level was set at $p<0.05$.

RESULTS

Intraplantar formalin injection (5%) in rats elicited characteristic biphasic nociceptive behaviors, comprising both supraspinally integrated responses (licking/lifting of the injected paw) and spinal reflex behaviors (rapid paw withdrawal).

Prophylactic (pre-treatment) effects: Control animals (PBS pre-treatment+formalin) displayed a typical peak in nociceptive behaviors immediately following formalin injection. Pre-treatment with either dose of the AMPA/kainate receptor antagonist CNQX (1 or 10 µM) did not induce statistically significant changes in licking/lifting duration or withdrawal reflex counts compared to the control group ($p>0.05$) (Fig. 1(a-b)). In contrast, pre-treatment with the NMDA receptor antagonist MK-801 (1 µM) produced a significant 65.7% reduction in the duration of licking/lifting behavior during the 5-10 min interval (Phase 1) compared to control ($p<0.05$) (Fig. 1b), although it did not significantly alter withdrawal counts (Fig. 1a).

Pre-treatment with *P. bistriata* crude venom (0.3 mg) resulted in a robust and broader antinociceptive profile. It significantly reduced licking/lifting behavior, with an average

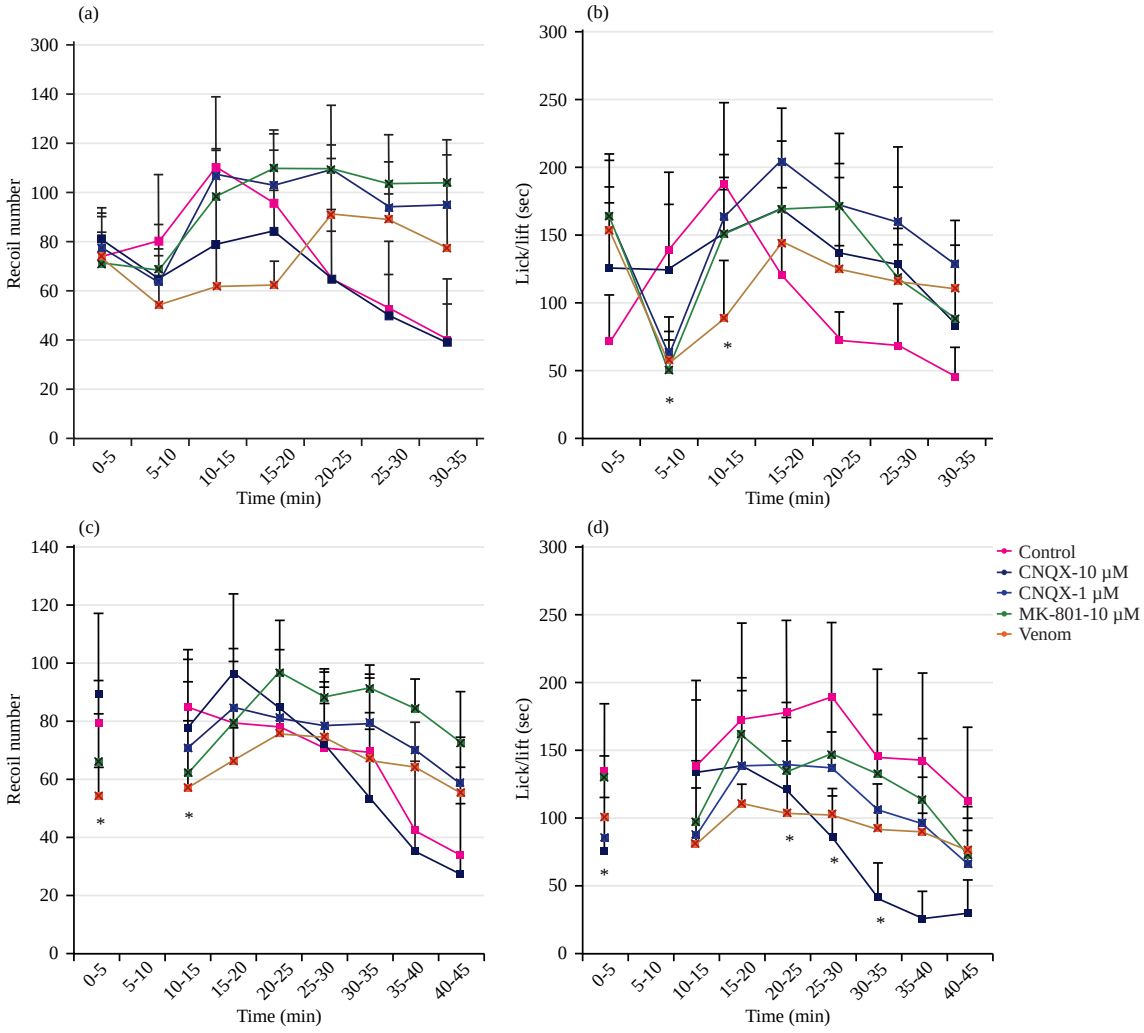


Fig. 1(a-d): Behavioral responses in Wistar rats after formalin injection, (a) Recoil behavior (pre-treatment): *P. bistriata* venom (20 mg/mL) significantly reduced responses at 10-15 min vs. control, (b) Licking/lifting (pre-treatment): MK-801 (1 µM) and venom reduced responses at 5-10 min; venom also reduced at 10-15 min vs. control, (c) Withdrawal behavior (post treatment): Venom reduced responses at 0-5 min and 10-15 min vs. control (5-10 min interval not analyzed) and (d) Licking/lifting (post-treatment): CNQX (10 µM) reduced responses at 0-5 min and from 25 min onward; venom reduced at 20-25 min vs. control (5-10 min interval not analyzed)
All results were analyzed using Kruskal-Wallis and Friedman tests, $p<0.05$

reduction of 56.6% from 5 to 15 min post-formalin compared to the control group ($p<0.05$) (Fig. 1b). Furthermore and uniquely among the prophylactic treatments, venom significantly attenuated the withdrawal reflex. Withdrawal counts were reduced starting at 5 min, with a 44.8% decrease becoming statistically significant during the 10-15 min interval ($p<0.05$) (Fig. 1a).

Therapeutic (post-treatment) effects: In control animals, nociceptive behaviors began to decline 25-30 min after formalin injection, corresponding to the late phase of the formalin model. Post-treatment with CNQX at 8 min produced a complex, dose-dependent effect. The high dose (10 µM) initially increased licking/lifting (43.7% increase from 10-20 min) but subsequently caused a significant 55.1% reduction from 25-45 min (late Phase 2) compared to control ($p<0.05$) (Fig. 1d). Withdrawal behavior was not significantly altered by CNQX at either dose (Fig. 1c). Post-treatment with MK-801 significantly reduced licking/lifting duration in the later phase (Fig. 1d) but, again, did not affect withdrawal counts (Fig. 1c).

Post-treatment with *P. bistriata* venom also modulated the response in a temporally complex manner. An initial increase in licking/lifting (27.3%, 15-20 min) was followed

by a significant 44.4% reduction from 20-30 min ($p<0.05$) (Fig. 1d). Importantly, venom post-treatment significantly reduced withdrawal behavior, specifically during the 10-15 min interval ($p<0.05$) (Fig. 1c), demonstrating a therapeutic effect on the spinal reflex not observed with the selective antagonists.

DISCUSSION

This study provides the first evidence for the peripheral antinociceptive activity of *P. bistriata* spider venom. The venom exhibited a distinct and broader pharmacological profile than the selective glutamatergic antagonists MK-801 and CNQX, effectively attenuating both the complex (licking/lifting) and reflexive (withdrawal) nociceptive behaviors induced by formalin in both prophylactic and therapeutic paradigms. The superior and consistent profile of the crude venom likely stems from its complex composition, which extends beyond simple glutamatergic modulation.

The selective reduction of supraspinally-mediated behavior by MK-801 underscores the importance of peripheral NMDA receptors and glutamatergic tone in the initial nociceptive burst of Phase 1[21-23], suggesting that peripheral NMDA receptor activation contributes specifically to the conscious sensory-discriminative and affective components of acute pain. While the site of injection in our model is peripheral, the known Central Nervous System (CNS) mechanisms of its isolated components inform a relevant synergistic hypothesis for peripheral activity. The acyl-polyamines Pwx1 and Pwx2, previously isolated from this venom, exhibit complementary actions: Pwx1 enhances glutamate clearance from the synaptic cleft[14,15], which would reduce excitatory tone, while Pwx2 is a potent inhibitor of GABA and glycine uptake[16,17], thereby potentiating inhibitory neurotransmission. This dual action, simultaneously reducing excitation and enhancing inhibition, illustrates a potent and complementary strategy inherent to the venom's cocktail that single-receptor antagonists cannot replicate. Such synergy is particularly relevant as impaired GABAergic inhibition is a recognized factor in pain states[24] and boosting inhibition via uptake blockade offers a distinct therapeutic angle that could operate at peripheral terminals or dorsal root ganglia, where GABA receptors are also expressed.

The venom's distinct ability to suppress both behaviors, unlike the selective glutamatergic antagonists, suggests a more comprehensive inhibitory action on the nociceptive pathway. This likely involves disruption of signal transmission not only at peripheral terminals but also within spinal circuits required for the reflex arc, potentially through simultaneous modulation of excitatory and inhibitory neurotransmission. The paradoxical biphasic effect of CNQX (initial pronociception followed by delayed analgesia) may reflect differential roles of AMPA/kainate receptors at different stages of peripheral sensitization or dose-dependent recruitment of modulatory circuits. Phase 2 of the formalin model involves peripheral inflammation and central sensitization maintained by ongoing peripheral input[3,5]. The venom's efficacy during this phase suggests it can dampen this sustaining drive, possibly by interfering with the inflammatory cascade or the sensitized state of peripheral nociceptors. While CNQX showed only late-phase effects limited to licking/lifting and MK-801 affected only the supraspinal component, the venom uniquely reduced both behaviors during Phase 2, further highlighting its multi-target therapeutic potential.

Our findings align with the historical success of multi-component natural products in pain management and the emerging paradigm of multi-target analgesics[11,25]. While selective antagonists are invaluable tools for dissecting mechanism, their therapeutic window can be narrow due to compensatory mechanisms or receptor subtype complexities. The *P. bistriata* venom, as a natural "cocktail", appears to engage multiple complementary pathways, potentially leading to greater efficacy and a wider safety margin, a hypothesis supported by the lack of motor impairment observed in previous central studies with its isolated acyl-polyamines[19].

CONCLUSION

This study provides the first evidence that peripheral administration of *P. bistriata* crude venom produces significant antinociceptive effects in both prophylactic and therapeutic paradigms of the formalin model. Unlike the selective glutamatergic antagonists MK-801 and CNQX, the venom uniquely attenuated both supraspinally-integrated pain behaviors (licking/lifting) and spinal reflex responses (withdrawal), suggesting a broader, multi-target mechanism of action. These findings support the therapeutic potential of this venom's complex pharmacological cocktail for pain management. Future studies should investigate isolated venom components in this peripheral model and explore their specific mechanisms on primary afferent neurons and local inflammatory mediators.

SIGNIFICANCE STATEMENT

This study provides the first evidence that *Parawixia bistriata* crude venom exerts peripheral antinociceptive effects in the formalin model, demonstrating broader analgesic activity than selective glutamatergic antagonists. These findings highlight the venom as a promising source of multi-target analgesic compounds and provide a foundation for the development of novel non-opioid pain therapeutics.

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