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Kappa-opioid receptor blockade in the inferior colliculus of prey threatened by pit vipers decreases anxiety and panic-like behaviour

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Abstract

The dorsal midbrain comprises dorsal columns of the periaqueductal grey matter and corpora quadrigemina. These structures are rich in beta-endorphinergic and leu-enkephalinergic neurons and receive GABAergic inputs from substantia nigra pars reticulata. Although the inferior colliculus (IC) is mainly involved in the acoustic pathways, the electrical and chemical stimulation of central and pericentral nuclei of the IC elicits a vigorous defensive behaviour. The defensive immobility and escape elicited by IC activation is commonly related to panic-like emotional states. To investigate the role of κ-opioid receptor of the IC in the antiaversive effects of endogenous opioid receptor blockade in a dangerous situation, male Wistar rats were pretreated in the IC with the κ-opioid receptor-selective antagonist nor-binaltorphimine at different concentrations and submitted to the non-enriched polygonal arena for a snake panic test in the presence of a rattlesnake and, after 24 h, prey were resubmitted to the experimental context. The snakes elicited in prey a set of antipredatory behaviours, such as the anxiety-like responses of defensive attention and risk assessment, and the panic-like reactions of defensive immobility and either escape or active avoidance during the elaboration of unconditioned and conditioned fear-related responses. Pretreatment of the IC with microinjections of nor-binaltorphimine at higher concentrations significantly decreased the frequency and duration of both anxiety- and panic-attack-like behaviours. These findings suggest that κ-opioid receptor blockade in the IC causes anxiolytic- and panicolytic-like responses in threatening conditions, and that kappa-opioid receptor-selective antagonists can be a putative coadjutant treatment for panic syndrome treatment.

Significant outcomes

- Nor-binaltorphimine at higher concentrations in the inferior colliculus (IC) decreases anxiety in an aversive condition.
- Nor-binaltorphimine at higher concentrations in the IC causes panicolytic-like behaviour.
- The selective κ-opioid receptor blockade in the IC attenuates panic attacks.
- The polygonal arena for snake panic test is a suitable experimental model to investigate novel drugs for panic attacks.

Limitations

- Only male experimental animals were submitted to the polygonal arena for snake panic test.
- The role of κ-opioid receptor in the antiaversive-like effect of nor-binaltorphimine was investigated only at dorsal midbrain caudal levels.

Introduction

The opioid neural system has been described to play a role in several functions in the central nervous system. There is a convergence of several neurochemical activities in the prefrontal cortex (Cole *et al.*, 2024) and in brainstem nuclei (Welsch *et al.*, 2023) regulating reward, motivational behaviour, affective behaviour, decision-making and cognitive control, adaptation to aversive/stressful situations (van Steenbergen *et al.*, 2019; Cole *et al.*, 2024), and affective and sensory aspects of pain (Gomtsian *et al.*, 2018; Cahill *et al.*, 2022), highlighting the potential therapeutic of endogenous opioid modulatory drugs for the treatment of psychiatric disorders. Psychological stressors, such as the nearby presence of a predator, can be strong enough to induce critical neurochemical alterations, changing the behavioural strategy in several species. However, little is known about how threats can alter the activity of the limbic system when the endogenous opioid receptors are either activated or inhibited. Downregulation of enkephalin in the lateral hypothalamus seems to be critical for inhibiting the neuronal activity and behavioural responses after the exposure to predator scent stimulus (You *et al.*, 2023). A decreased μ-opioid receptor in the amygdaloid complex of monkeys with self-injurious behaviour and reduced prodynorphin in the hypothalamus were recently reported (Jackson *et al.*, 2023). There is evidence that dynorphyn/kappa-opioid receptor signalling can potentially modify subcortical function though a kappa-opioid receptor-driven inhibition of GABAergic activity (Pina *et al.*, 2020) and kappa-opioid receptor antagonists have a putative therapeutic effect for treatment of mental disorders (Varastehmoradi *et al.*, 2020).

In addition, the endogenous opioid system plays a crucial role in regulating innate fear-related behaviours triggered by electrical and chemical stimulation of the dorsal midbrain in both cranial (Coimbra *et al.*, 1996; Eichenberger *et al.*, 2002) and caudal mesencephalon (Cardoso *et al.*, 1992; Coimbra *et al.*, 2000; Calvo and Coimbra, 2006; Castellan-Baldan *et al.*, 2006). Evidence suggests the presence of κ-opioid receptors in forebrain structures such as the hippocampus, cerebral frontal lobe cortex, neostriatum, and dorsal thalamus (Csillag *et al.*, 1990; Drake *et al.*, 1996; Sojka *et al.*, 2022), in both dorsal (Gutstein *et al.*, 1998) and ventral (Foote and Maurer, 1983) midbrain across different species.

Despite the acknowledged role of the hypothalamus in defensive behaviour (de Freitas *et al.*, 2013), the midbrain aversion system encompasses the periaqueductal grey matter (PAG), deep layers of the superior colliculus (dlSC), and the inferior colliculus (IC) (Cardoso *et al.*, 1992; Coimbra and Brandão, 1993; Coimbra *et al.*, 2006; Roncon *et al.*, 2013; de Mello Rosa *et al.*, 2022; Reis *et al.*, 2023). The IC consists in a brainstem structure critically involved in the elaboration of defensive responses (Melo *et al.*, 1992; Brandão *et al.*, 1993; Melo and Brandão, 1995a, b) and active avoidance learning (Brandão *et al.*, 1997) in addition to the processing of acoustic stimuli. Stimulation of dorsal columns of PAG (dPAG), dlSC, and IC specifically induces defensive attention, defensive immobility, and escape behaviour akin to prey responses when facing predators (Dean *et al.*, 1989; Brandão *et al.*, 2005; Lobão-Soares *et al.*, 2008; Almada and Coimbra, 2015; Almada *et al.*, 2015; de Paula Rodrigues *et al.*, 2024; Falconi-Sobrinho *et al.*, 2024). Dynorphin-containing pathways from the striatum to the substantia nigra pars reticulata (SNpr) appear to modulate SNpr neuronal activity, leading to the inhibition of panic-related emotions (Castellan-Baldan *et al.*, 2006; Almada *et al.*, 2021; da Silva *et al.*, 2023).

Morphological evidence points to interactions between opioid peptides and γ-aminobutyric acid (GABA)-labelled perikarya in the central and pericentral nuclei of the IC (Tongjaroenbuangam *et al.*, 2006). Pharmacological findings suggest that opioid peptide-GABA interactions in the dorsal midbrain tectum reduce unconditioned fear-induced behaviour (Eichenberger *et al.*, 2002). Furthermore, the opioid receptor blockade in the ventral midbrain demonstrates panicolytic-like effects, lowering the threshold for escape behaviour (Ribeiro *et al.*, 2005; da Silva *et al.*, 2013). The opioid system is implicated in modulating panic attack-like defensive reactions in laboratory animals subjected to the polygonal arena for snake panic test (Coimbra *et al.*, 2017a; Calvo *et al.*, 2019b; Calvo *et al.*, 2019b). The pharmacological and ethological validations of enriched (Uribe-Mariño *et al.*, 2012; Almada RC *et al.*, 2021; de Paula-Rodrigues *et al.*, 2024) and non-enriched polygonal arenas (Lobão-Soares *et al.*, 2008; Coimbra *et al.*, 2017a) as suitable aversive environments (Falconi-Sobrinho *et al.*, 2023) for assessing the effects on limbic system structures of new potential drugs with antiaversive activity (Twardowschy *et al.*, 2013; Paschoalin-Maurin *et al.*, 2018; Calvo *et al.*, 2019c) have been recently established by our team (Paschoalin-Maurin *et al.*, 2018).

Despite the abundant evidence supporting the role of midbrain tectum endogenous opioid peptides in modulating unconditioned fear-related defensive responses, this study aims to explore whether the specific blockade of κ-opioid receptors in the IC with the κ-opioid receptor-selective antagonist nor-binaltorphimine affects the organisation of anxiety- and panic attack-like responses exhibited by prey in the non-enriched polygonal arena for snake panic test.

Material and methods

Animals

Formal approval for all experiments was obtained from the Commission of Ethics in Animal Experimentation of the FMRP-USP (processes 064/2004 and 205/2008), adhering to the ethical principles in animal research established by the Brazilian College of Animal Experimentation (COBEA) and the National Council for Animal Experimentation Control (CONCEA). Male Wistar rats ($N = 48$), aged 9 to 11 weeks and weighing 200 – 250 g, were sourced from the animal facility at the University of São Paulo Ribeirão Preto School of Pharmaceutical Sciences (FCFRP-USP) and used in groups of four, housed in plastic boxes (40 × 33 × 26 cm) for a minimum of 48 h before the commencement of the experiment. The rats were maintained under controlled conditions (23 ± 1°C; 12-h/12-h light/dark cycle, lights on at 7 a.m.), with free access to food and water.

The predators employed were wild male and female venomous snakes (*Crotalus durissus terrificus,* Reptilia, Viperidae), weighing 618, 702, 1038, and 1232 g ($N = 4$) fed in intervals of 15 days. These rattlesnakes were sourced from the Brazilian Southeast rainforests and housed in the ophidiarium of the animal house at the School of Medicine of Ribeirão Preto of the University of São Paulo (FMRP-USP). This facility is licensed by the Brazilian government (IBAMA Committee process 1/35/1998/000846-1). One week before the experiments, the rattlesnakes were kept in a sun-lit field with appropriate shelter, grass, and water sources in the Laboratory of Neuroanatomy and Neuropsychobiology of the Ribeirão Preto Medical School of the University of São Paulo (LNN-FMRP-USP)/Behavioural Neurosciences Institute (INeC) ophidiarium. This ophidiarium is licensed by both the Brazilian government (IBAMA 3543.6986/2012-SP and 3543.6984/2012-SP processes) and the São Paulo State government (SMA/DeFau 15.335/2012 process;

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MEDUSA Project, SISBIO authorisation for activities with scientific purposes 41435-1, 41435-2, and 41435-4 processes; SIGAM authorisation of installation process 39.043/2017; SIGAM authorisation for use and handling of wild snakes process 39.044/2017).

The serpent enclosure at the LNN-FMRP-USP is illuminated by natural sunlight and includes artificial waterfalls, natural rocks, and a combination of natural and artificial tropical plants. It is maintained under a light/dark cycle of 12/12 h (lights on from 7 a.m. to 7 p.m.) and at a constant room temperature of 27°C ± 1°C (60–70% humidity). In this experiment, the rattlesnakes were fed at two additional specific times: 24 h prior (with previously euthanised rats in a CO_2 chamber) and immediately before the commencement of each experiment with live *Rattus norvegicus*.

Surgery

The rats underwent anaesthesia using ketamine at a dosage of 92 mg/kg (Ketamine Agener®, União Química Farmacêutica Nacional, Brazil; 0.2 ml of 10% solution) and xylazine at 9.2 mg/kg (Dopaser®, Hertape/Calier, Juatuba, Minas Gerais, Brazil) and were secured in a stereotaxic frame (David Kopf, Tujunga, California, USA). The upper incisor bar was positioned 3.3 mm below the interaural line, ensuring a horizontal alignment of the skull between bregma and lambda. Unilateral implantation of guide cannulae (o.d. 0.6 mm, i.d. 0.4 mm) for drug injections into the IC was performed. The guide cannulae were inserted vertically using the following coordinates, with bregma as the reference point: anterior/posterior, −8.6 mm; medial/lateral, ±1.2 mm; dorsal/ventral, −4.3 mm (Paxinos & Watson, 2007). Acrylic resin and two stainless-steel screws were used to secure the cannulae to the skull. Each guide cannula was safeguarded with a stainless-steel wire to prevent obstruction. Subsequently, each rat received an intramuscular injection of penicillin G-benzathine (120 000 UI; 0.2 ml) followed by an intramuscular injection of the analgesic and anti-inflammatory drug flunixin meglumine (2.5 mg/kg). Following the surgical procedure, the rats were given a recovery period of 5 days.

Drugs

The selection of drugs, their respective doses, and the timing of injections were informed by prior investigations conducted in our laboratory (da Silva *et al.*, 2013; Calvo *et al.*, 2019a; Osaki *et al.*, 2003). The opioid antagonist norbinaltorphimine (nor-BNI, Sigma Chemical Co., St Louis, USA) was dissolved in saline (NaCl; 0.9%) immediately prior to administration. Microinjections of 1, 3, and 5 µg were delivered at a constant volume rate (0.2 µl/min) into the IC, and behavioural responses were documented 2 h post the pharmacological treatment (Ling *et al.*, 1986; Osaki *et al.*, 2003).

Non-enriched polygonal arena for snake panic test

To facilitate prey versus predator confrontations, we utilised a semi-transparent acrylic enclosure comprising a high-walled transparent acrylic rectangular parallelepiped-shaped polygonal arena (154 × 72 × 64 cm). The inner walls were covered with a reflective film, ensuring 80 – 90% light reflection to minimise visual contact between the predator and the surrounding experimental area. This design aimed to focus the attention of the predator on the experimental rat. The arena floor was divided into 20 equal rectangles by green fluorescent lines drawn with a marker pen (4.2 mm width; Pritt mark-it). As described in previous papers from our laboratory (Coimbra *et al.*, 2017a; Paschoalin-Maurin *et al.*, 2018), this non-enriched environment for prey versus snake confrontations served as an experimental model for panic attacks.

Each rat received microinjections of either nor-BNI or physiological saline into the IC two hours before the behavioural test. On the experimental day, the snake was carefully placed in one corner of the polygonal arena, and the rats were gently captured with a nylon net and placed diagonally opposite to the rattlesnake. After 15 min of confrontation, the rodent was removed, and the snake occupied the opposite corner. The next rat was introduced in the corner vacated by the predator, repeating the prey versus predator confrontation during the same period, alternating the positions of each tested rat. Twenty-four hours later, rats were re-exposed to the experimental context without additional pharmacological treatment in the absence of a snake for 15 min. Behaviours were recorded using a videocamera (Sony Handcam HDR-CX350, Konan, Minato-ku, Tokyo, Japan), enabling a blind ethological analysis by the researcher.

Rodent behaviours were quantified as follow (Coimbra *et al.*, 2017a): defensive attention (alertness), operationalised as the interruption of ongoing behaviour with occasional scanning of the environment; stretched attend posture, where the rat stretches to its full length with forepaws and hind paws in the same place; flat back approach, defined as forward elongation of the body; startle, a sudden involuntary movement elicited by something frightening; defensive immobility (freezing), defined as immobility for at least 6 s in a dorsiflexion defensive posture; escape, running or jumping away from the predator; active avoidance, fast locomotor behaviour in different directions from those in which the predator was situated during the previous confrontation.

Predatory and defensive behaviours displayed by the snakes included: (I) threatening postures, (II) threatening attacks, (III) offensive/defensive attacks, (IV) defensive postures, (V) predatory attacks, and (VI) crossing, which involved body movements through four delimited rectangles in the arena floor after crossing the section lines.

Experimental groups: (a) Nor-binaltorphimine at 5 µg/ 0.2 µl (IC) + No threat ($n = 8$); re-exposure to the experimental context after 24 h; (b) Physiological Saline (IC) + No threat ($n = 8$); re-exposure to the experimental context after 24 h; (c) Physiological Saline (IC) + Snake confrontation ($n = 8$); re-exposure to the experimental context after 24 h; (d) Nor-binaltorphimine at 1 µg/0.2 µl (IC) + Snake confrontation ($n = 8$); re-exposure to the experimental context after 24 h; (e) Nor-binaltorphimine at 3 µg/0.2 µl (IC) + Snake confrontation ($n = 8$); re-exposure to the experimental context after 24 h; (f) Nor-binaltorphimine at 5 µg/0.2 µl (IC) + Snake confrontation ($n = 8$); re-exposure to the experimental context after 24 h.

Psychobiological experiment

Considering that morphological attributes of the predator, such as biological body mass, rattle size, and threatening behaviour, may potentially evoke varying degrees of antipredatory responses in prey, independent groups of naïve Wistar rats ($n = 8$ per group) underwent exposure to four rattlesnakes with distinct characteristics following habituation in the polygonal arena for snake panic test. The characteristics of the snakes were as follows: snake S1, weighing 1232 g, possessed the longest rattle and exhibited high motility; snake S2, weighing 1038 g, had a medium-sized rattle and displayed low motility; snake S3, weighing 702 g, featured a medium-sized rattle and demonstrated low motility; snake S4, weighing 618 g, had the shortest rattle and showed high motility.

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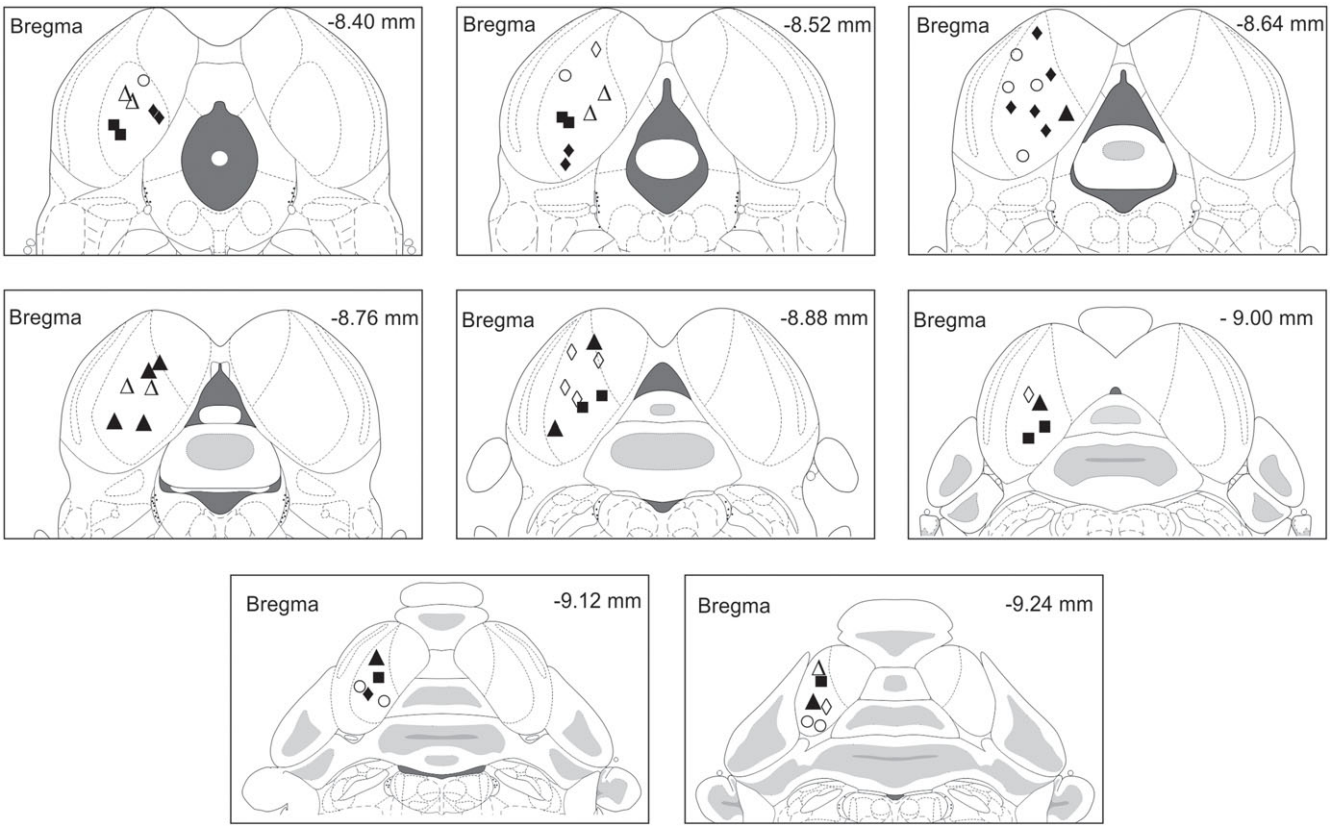

Figure 1. Schematic representation of *Rattus norvegicus* midbrain transverse sections, showing histologically confirmed sites of intracollicular microinjections of either nor-binaltorphimine (nor-BNI) at 5.0 μg/0.2 μl (◇) or physiological saline (Δ) in non-threatened group, and either physiological saline (◆), nor-BNI at 1.0 μg/0.2 μl (■), 3.0 μg/0.2 μl (■) or 5.0 μg/0.2 μl (▲) in inferior colliculus of threatened rats depicted in diagrams from Paxinos and Watson´s rat brain in stereotaxic coordinates atlas (2007).

Prey versus predator confrontations were recorded over 5 min inside the non-enriched polygonal arena for snakes panic test.

Histology

The rats underwent anesthesia with ketamine (92 mg/kg) and xylazine (9.2 mg/kg), followed by transcardial perfusion with ice-cold phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA, pH 7.3). The perfusion was carried out through the left cardiac ventricle using a perfusion pump (Master Flex® L/STM peristaltic tubing pump, East Bunker Court Vernon Hills, Illinois, USA). The brain of each rat was collected, fixed in 4% PFA for 24 h, and cryoprotected by immersion in 10% and 20% sucrose solutions for 24 h each. Coronal sections of 60 μm thickness were then cut using a cryostat (CM 1950 Leica, Wetzlar, Germany). Following this, the slices were carefully mounted on glass slides coated with chrome alum gelatin to prevent detachment. Subsequently, the sections were stained with methylene blue using a robotic autostainer (CV 5030 Leica Autostainer) to facilitate the identification of microinjection sites under light microscopy. The positions of microinjection sites were examined using an AxioImager Z1 motorised photomicroscope (Carl Zeiss Strasse, Oberkochen, Germany). Microinjections were administered within the IC, specifically in the central nucleus of the IC, as illustrated in diagrams modified from the Paxinos and Watson atlas, as depicted in Figure 1.

Statistics

All statistical analyses were conducted using GraphPad Prism (GraphPad Software Inc., California, USA). The normality of data from independent groups was assessed through the Shapiro–Wilk test. For psychobiological experiments, data were subjected to the Kruskal–Wallis ANOVA on ranks due to the absence of a Gaussian distribution, and results were presented as median and percentiles. In neuropsychopharmacological experiments, parametric tests were employed as the data adhered to Gaussian distributions and variances between groups were homogenous for over 50% of the data.

Control group data were analysed using independent samples Student's *t*-test, comparing the saline/no threat versus saline/threatened groups, as well as comparing the saline/no threat versus nor-BNI (5.0 μg)/no threat groups. Data obtained post-prey versus-rattlesnake confrontations and after prey exposure to the experimental context without the predator underwent one-way ANOVA. Treatment (different doses) served as the main factor, and Dunnett's *post hoc* test was utilised to compare each drug dose with the vehicle-treated control group within each condition (unconditioned and conditioned fear). Results are presented as the mean + standard error of the mean (S.E.M.), with values of $p < 0.05$ considered statistically significant.

Results

Psychobiological analysis of rattlesnakes versus prey behaviour

In a psychobiological study investigating prey reactions based on different biological masses, rattle sizes, and threatening behaviours of the predator, separate groups of naïve Wistar rats were exposed to each rattlesnake, as illustrated in Figure 2. All prey consistently exhibited antipredatory behaviour in the presence of the potential

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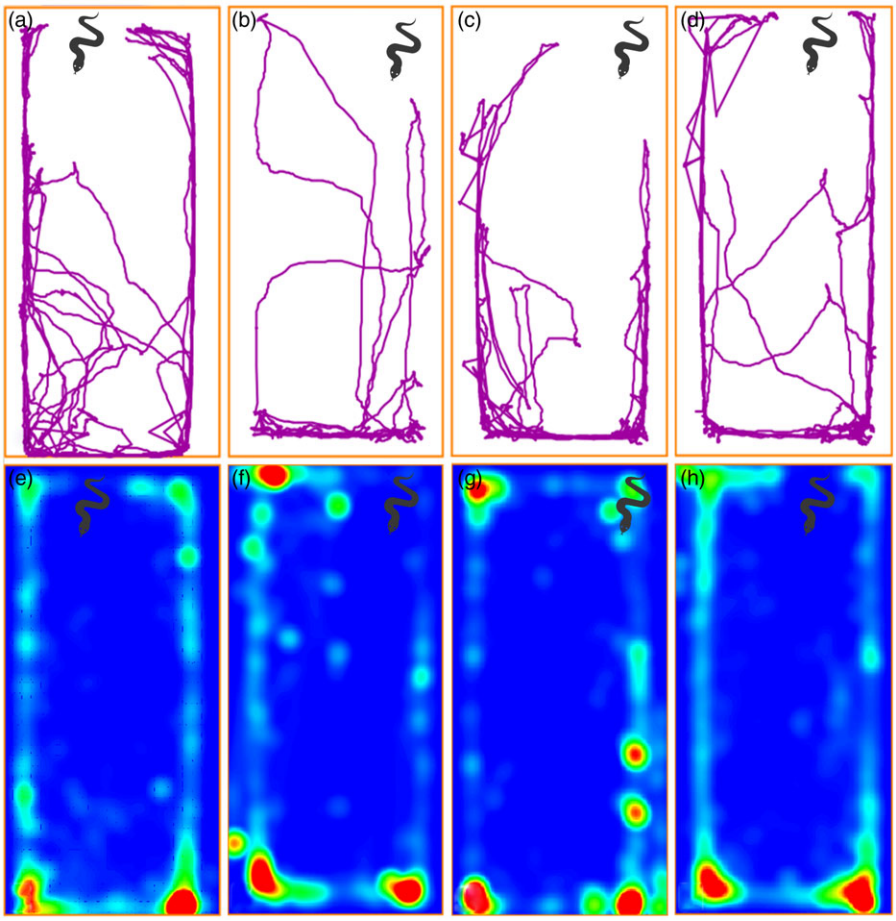

Figure 2. Antipredatory behaviour displayed by naïve *Rattus norvegicus* confronted by *Crotalus durissus terrificus* venomous pit vipers with different morphological and behavioural characteristics in the polygonal arena for snakes panic test. A – H: locomotor/place preference behaviour displayed by prey in the presence of either snake S1, weighing 1232 g, with the longest rattle and high motility (A and E); snake S2, weighing 1038 g, with a medium size rattle and presenting low motility (B and F); snake S3, weighing 702 g, with a medium size rattle, presenting low motility (C and G); snake S4, weighing 618 g, with the shortest rattle, and presenting high motility (D and H), recorded by Anymaze software.

predator, exploring all areas of the aversive environment while actively avoiding the position of the rattlesnake, as depicted in Figure 2A–H. The behaviours of each predator are depicted in Table 1. Rattlesnake S2 demonstrated the highest frequency of threatening postures (36%), rattle vibration (9%), offensive strikes (8%), and defensive strikes (12%).

Interestingly, distinct groups of prey confronted by each rattlesnake did not exhibit significant differences in the incidence/duration of defensive attention ($H_{(2)} = 0.397$; $p > 0.05/H_{(2)} = 3.194$; $p > 0.05$), nor in the incidence of stretch attend posture ($H_{(2)} = 3.723$; $p > 0.05$), flat back approach ($H_{(2)} = 3.95$; $p > 0.05$), startle ($H_{(2)} = 5.677$; $p > 0.05$), or escape behaviour ($H_{(2)} = 1.04$; $p > 0.05$), as depicted in Table 2. However, the more intense aversive stimuli presented by rattlesnake S2 resulted in a significant increase in the incidence ($H_{(2)} = 21.27$; $p < 0.05$) and duration ($H_{(2)} = 20.85$; $p < 0.05$) of defensive immobility in prey, as shown in Table 2. Consequently, rattlesnake S2 was not included in the psychopharmacological experiments to standardise predator behaviour.

Defensive behavioural responses displayed by Crotalus durissus terrificus *pit vipers during the confrontation with Wistar rats and prey antipredatory behaviour*

In the psychopharmacological experiments, prey confronted with rattlesnakes exhibited notable antipredatory reactions, including defensive attention (Figure 3A and D), defensive immobility (Figure 3B), escape behaviour (Figure 3C), flat back approach (Figure 3E), and interaction with the predator (Figure 3F). Throughout the prey versus snake confrontation tests conducted in the non-enriched polygonal arena for snakes panic test, all pit vipers exhibited attentional behaviour towards the prey. As depicted in Figure 4A, they assumed a threatening posture with the elevation of the head and anterior body region (32.42%), often followed by tail vibration. Threatening attacks (4.36%) and defensive postures (22.9%) – wherein the snake retracted the anterior portion of its body in a sigmoid curve, posed for a potential strike (as illustrated in Figure 3D) – were also observed. Threatening rattle vibration (22.71%) served as a warning behavioural response, and the snakes commonly retreated backwards with a threatening/defensive posture when confronted with fearless prey. Offensive/defensive strikes involving biting occurred at an incidence of only 3.92%. In the absence of pit vipers, when exposed to the experimental context, Wistar rats displayed defensive attention, defensive immobility, and active avoidance, as depicted in Figures 5 and 6.

Unconditioned fear-induced behaviour elicited by rats threatened by rattlesnakes

Rats exposed to threats from rattlesnakes exhibited notable increases in the expression of defensive attention (number: $t_{15} = 2.29$; $p < 0.001$; duration: $t_{15} = 3.81$; $p < 0.01$), as well as in the incidence of flat back approach ($t_{15} = 5.77$; $p < 0.001$) and startle responses ($t_{15} = 4.89$; $p < 0.001$), as illustrated in Figure 5 (A–D). Additionally, there was a significant rise in the expression of defensive immobility (number: $t_{15} = 8.30$; $p < 0.001$; duration: $t_{15} = 4.73$; $p < 0.001$) and escape response (number: $t_{15} = 5.40$; $p < 0.001$; duration: $t_{15} = 5.27$), as depicted in Figure 5 (A–D).

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Table 1. Incidence (in percentage) of threatening posture, defensive posture, rattle vibration, offensive strike, defensive strike, and crossings displayed by different rattlesnakes

	Behavioural incidence (%)			
Snake behaviour	S1	S2	S3	S4
Threatening posture	12	36	4	0
Defensive posture	52	31	90	100
Rattle vibration	6	9	3	0
Offensive attack	0	8	0	0
Defensive attack	0	12	0	0
Crossings	32	4	3	0

Table 2. Incidence and duration of defensive/antipredatory behavioural responses, such as defensive attention, stretch attend posture, flat back approach, startle, prey versus predator interaction, escape behaviour, and defensive immobility (freezing) displayed by prey in the polygonal arena for snakes panic tests in the presence each rattlesnake. Data are represented as the 25th percentile, median, and 75th percentile. *Significant difference ($p < 0.05$) as compared to the other groups, according to Kruskal–Wallis H nonparametric test, followed by Dunn's *post hoc* test

Rattus norvegicus behaviour	S1	S2	S3	S4
Number of defensive attention	0.88; 0.9; 1.22	0.74; 0.9; 1.37	0.59; 1.01; 1.37	0.63; 1.01; 1.45
Duration of defensive attention	1.95; 2.43; 2.82	1.26; 2.74; 3.64	2.64; 3.19; 3.61	2.60; 2.65; 3.04
Stretch attend posture	1.47; 1.71; 1.88	1.23; 1.53; 1.68	1.52; 1.81; 1.88	1.57; 1.73; 1.86
Flat back approach	2.04; 2.34; 2.62	1.53; 2.46; 2.65	1.94; 2.61; 2.65	2.48; 2.64; 2.66
Startle	0.23; 0.43; 0.54	0.2; 0.31; 0.64	0.12; 0.21; 0.23	0.18; 0.23; 0.49
Prey-predator interaction	0.75; 0.94; 1.18	0.67; 0.84; 1.23	0.77; 1; 1.45	0.78; 1; 1.35
Escape	1.37; 1.49; 1.60	1.34; 1.36; 1.5	1.36; 1.45; 1.58	1.35; 1.5; 1.63
Number of defensive immobility	0	0.82; 1.17; 1.39*	0; 0; 0.17	0
Duration of defensive immobility	0	0.14; 1.65; 2.33*	0; 0; 0.75	0

Effect of IC κ-opioid receptors blockade with nor-binaltorphimine (1.0, 3.0 and 5.0 μg) on defensive behaviour elicited by rats threatened by rattlesnakes (unconditioned fear)

According to the one-way ANOVA, there was a significant effect of the treatment on the number ($F_{3,36} = 20.94$, $p < 0.001$) and duration ($F_{3,36} = 12.27$, $p < 0.001$) of defensive attention during the prey versus predator paradigm (unconditioned fear). The IC treatment with nor-binaltorphimine at higher doses (3.0 μg and 5.0 μg) significantly decreased the number (Dunnett's *post hoc* test; $p < 0.01$ and $p < 0.001$, respectively) of defensive attention, and the IC nor-BNI 1.0 μg-, 3.0 μg-, and 5.0 μg-treated groups decreased the duration (Dunnett's *post hoc* test; $p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively) of defensive attention behaviour, as illustrated in Figure 5A and B.

Concerning flat back approach and startle defensive behaviours, the one-way ANOVA showed a significant effect of the treatment ($F_{3,36} = 14.85$ and $F_{3,36} = 10.34$, respectively; $p < 0.001$ in both cases). IC treatment with nor-binaltorphimine at higher doses (3.0 μg and 5.0 μg) significantly decreased (Dunnett's *post hoc* test; $p < 0.001$ in both cases) the number of flat back approach, and nor-BNI 1.0 μg-, 3.0 μg-, and 5.0 μg-treated groups decreased the frequency of startle behaviours (Dunnett's *post hoc* test; $p < 0.001$, $p < 0.01$, and $p < 0.001$, respectively), as depicted in Figure 5C and D.

The one-way ANOVA also revealed a significant effect of the treatment on the number ($F_{3,36} = 12.92$, $p < 0.001$) and duration ($F_{3,36} = 11.59$, $p < 0.001$) of defensive immobility displayed by rats during unconditioned fear. The IC nor-BNI 3.0 μg- and 5.0 μg-treated groups decreased the number (Dunnett's *post hoc* test; $p < 0.05$ and $p < 0.001$, respectively) of defensive immobility, and only the treatment of the IC with nor-binaltorphimine at the highest dose (5.0 μg) significantly decreased the duration (Dunnett's *post hoc* test; $p < 0.001$) of defensive immobility, as shown in Figure 6A and B.

Furthermore, the one-way ANOVA indicated a significant effect of the treatment on the number ($F_{3,36} = 18.64$, $p < 0.001$) but not the duration ($F_{3,36} = 2.40$, $p > 0.05$) of escape behaviours displayed by threatened rats. The IC nor-BNI 3.0 μg- and IC nor-BNI 5.0 μg-treated groups significantly decreased the number (Dunnett's *post hoc* test; $p < 0.01$ and $p < 0.001$, respectively) of escape behaviour displayed by prey in the presence of rattlesnakes, as shown in Figure 6C and D.

Conditioned fear-induced behaviour elicited by rats during the re-exposure to the experimental context without the predator

Considering the irreversible long-lasting binding of nor-binaltorphimine on kappa-opioid receptors, the conditioned fear-induced behaviour was investigated 24 h after the intracollicular pretreatment with nor-binaltorphimine performed in the same rats previously threatened by the predator without new pharmacological treatment. Rats exposed to the experimental context without the rattlesnake exhibited a significant increase in the incidence of defensive attention (number, $t_{15} = 4.48$; $p < 0.001$;

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Figure 3. Defensive behaviour/Fearlessness displayed by *Rattus norvegicus* pretreated with intracolliculur microinjections of either physiological saline (A – C) or nor-binaltorphimine at 3.0 µg/0.2 µl and threatened by *Crotalus durissus terrificus* venomous pit vipers in the polygonal arena for snakes panic test. A and D: defensive attention. B: defensive immobility. C: escape behaviour. E: flat back approach. F: fearlessness prey versus predator interaction.

duration, $t_{15} = 3.43$; $p < 0.01$), as depicted in Figure 5A and B. Additionally, there was a notable increase in the incidence of defensive immobility (number, $t_{15} = 3.82$; $p < 0.01$; duration, $t_{15} = 7.16$; $p < 0.01$) and active avoidance responses (number, $t_{15} = 6.34$; $p < 0.001$; duration, $t_{15} = 5.72$; $p < 0.001$), as illustrated in Figure 5A–D.

Effect of IC κ-opioid receptors blockade with nor-BNI (1.0, 3.0, and 5.0 μg) on defensive behaviour elicited by rats during the re-exposure to the experimental context (conditioned fear)

After the intracollicular treatment of Wistar rats with a single dose of nor-binaltorphimine at different concentrations, in independent group of rodents, each previously threatened rat was submitted to a re-exposure to the experimental context 24 h after the interaction with the rattlesnake, without new pharmacological treatment. According to the one-way ANOVA, regarding the re-exposure of prey to the experimental context (conditioned fear) without the snake, there were significant effects of the treatment on the number ($F_{3,36} = 14.68$, $p < 0.001$) and duration ($F_{3,36} = 8.88$, $p < 0.001$) of defensive attention behaviour. The IC treatment with nor-binaltorphimine at 3.0 µg and 5.0 µg significantly decreased the number (Dunnett's *post hoc* test; $p < 0.001$ and $p < 0.05$, respectively) and duration (Dunnett's *post hoc* test; $p < 0.001$ in both cases) of defensive attention responses, as depicted in Fig. 5A and B. Neither flat back approach nor startle behaviours were displayed during the re-exposure of prey to the experimental context.

The treatment of the IC with nor-BNI at the highest dose (5.0 µg) significantly decreased the duration of defensive immobility ($F_{3,36} = 4.27$, $p < 0.05$, one-way ANOVA followed by Dunnett's *post hoc* test), as shown in Figure 5B. There was also a significant effect of the treatment on the number ($F_{3,36} = 14.4$, $p < 0.001$) and duration ($F_{3,36} = 5.14$, $p < 0.01$) of active avoidance behaviour. The treatment of the IC with nor-BNI at 1.0 µg and 5.0 µg significantly decreased the number of active avoidance (Dunnett's *post hoc* test; $p < 0.001$ in both cases), and the IC treatment with nor-BNI at 1.0 µg, 3.0 µg, and 5.0 µg significantly decreased the duration (Dunnett's *post hoc* test; $p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively) of active avoidance, as illustrated in Figure 6C and D.

Effect of IC κ-opioid receptors blockade with nor-BNI on motor behaviour

The IC administration of the κ-opioid receptor-selective antagonist nor-binaltorphimine did not induce motor impairments, as evidenced by the lack of statistically significant differences in the motor behaviours, such as crossings and rearing, between prey treated in the IC with nor-binaltorphimine at the highest concentration (5.0 µg/ 0.2 µl) and the non-threatened control group. This was observed both during exposure to the context ($t_{12} = 1.71$; $p > 0.05$ for crossing, and $t_{12} = 0.29$; $p > 0.05$ for rearing) and during re-exposure to the experimental context without the predator ($t_{12} = 1.31$; $p > 0.05$ for crossing, and $t_{12} = 0.10$; $p > 0.05$ for rearing), as shown in Figure 7.

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(a)

Snake Behaviour	Description	Percentage
Threatening posture	Elevation of the first third of the body in a sigmoid-shape	32.42%
Rattle movements	Continuous and rapid movements of the rattle	22.71%
Threatening strike	Strikes without a bite	4.36%
Offensive strike	Strikes with bite	3.92%
Defensive posture	Immobility with body in a coil-shape	22.9%
Crossing	Body movements through the border for the limited rectangles in the arena floor	28.75%

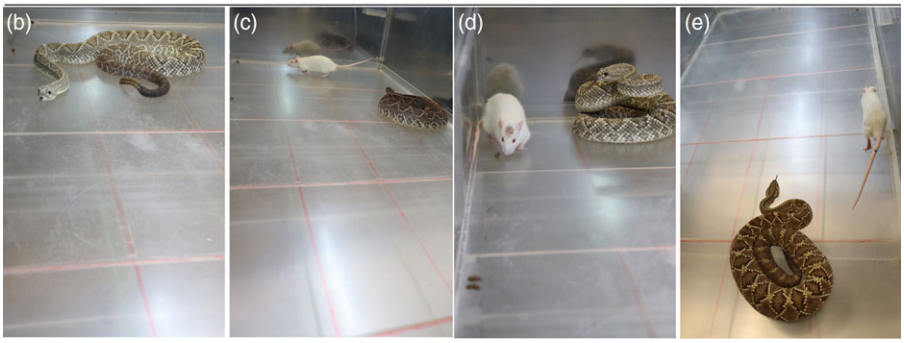

Figure 4. The incidence of behaviours displayed by venomous snakes (*Crotalus durissus terrificus*) during the confrontation with Wistar rats. Percentage of snake behaviour, during the 5-min encounter with rats treated with either physiological saline or nor-binaltorphimine at different concentrations in the inferior colliculus.

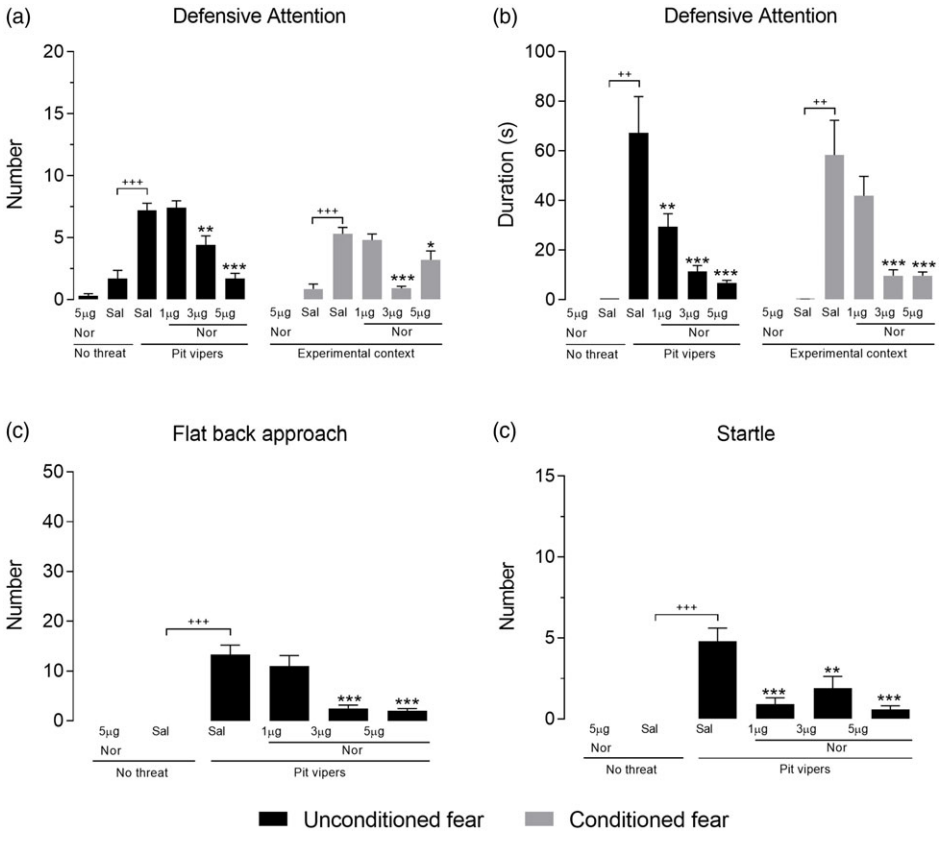

Figure 5. Effect of intracollicular pretreatment with saline (Sal), nor-binaltorphimine (nor-BNI; 1.0, 3.0 and 5.0 µg/0.2 µl) on the number (A) and duration (B) of defensive attention and number of flat back approach (C) and startle (D) exhibited by rats submitted to the confrontation with rattlesnakes in a polygonal arena (unconditioned fear) and to the experimental context without the predator (conditioned fear). Data are presented as mean + S.E.M., $n = 7, 7, 10, 10, 10$ and 10 for nor-BNI 5.0 µg/0.2 µl (No threat), Sal (No threat), Sal, nor-BNI at 1.0, 3.0 and 5.0 µg/0.2 µl (Threatened groups), respectively; $^{++}$, $p < 0.01$ and $^{+++}$, $p < 0.001$ comparing Sal-no threat group and Sal-threatened group (according student's *t*-test for independent samples); *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ compared to respective sal-treated group during the exposure of prey to the rattlesnakes (unconditioned fear) or the exposure of prey to the experimental context without the predator (conditioned fear), according to the one-way ANOVA, followed by Dunnett's *post hoc* test.

Discussion

All rodents exposed to the presence of rattlesnakes displayed a robust defensive behaviour, showing some anxiety-like responses, such as defensive attention and flat back approach (risk assessment behaviours), and panic attack-like reactions, such as defensive immobility and escape behaviour. There was an aetiological sequence of defensive behaviour displayed by potential prey, such as defensive attention, flat back approach and/or stretched attend posture, startle, defensive immobility, defensive interaction between prey and predator and escape behaviour followed by post-escape freezing (Falconi-Sobrinho *et al.*, 2023). However, not all prey displayed the whole range of these antipredatory behaviours, as previously reported by our team (Ferreira-Sgobbi *et al.*, 2022).

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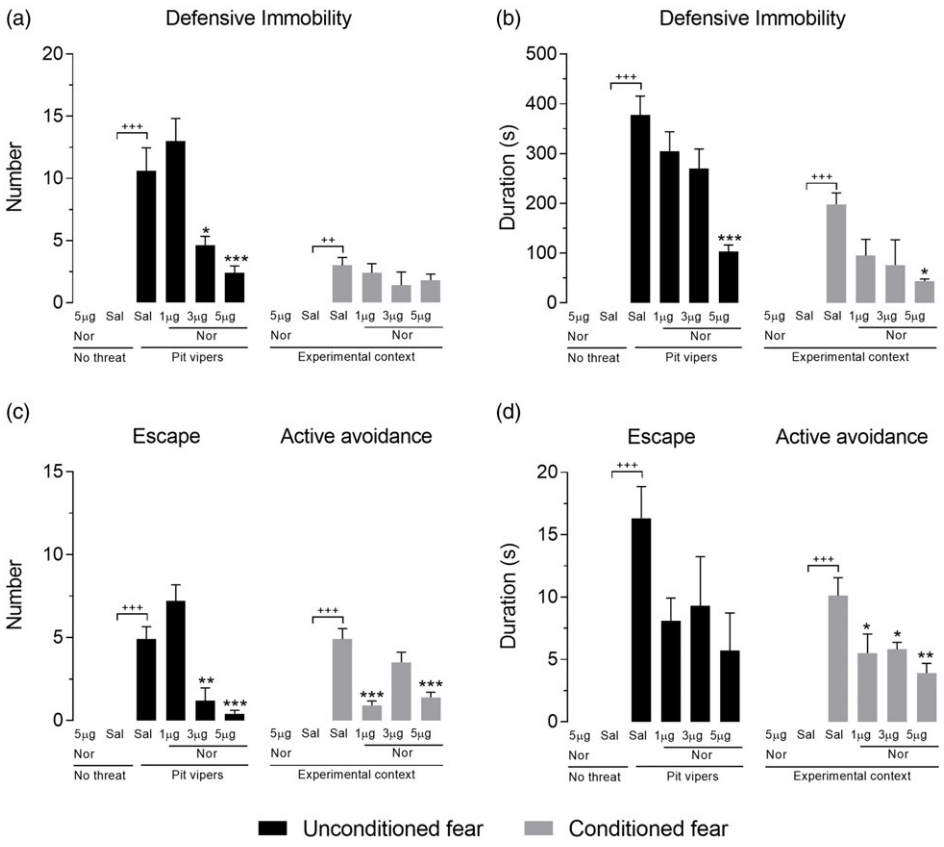

Figure 6. Effect of intracollicular pretreatment with saline (Sal), nor-binaltorphimine (nor-BNI; 1.0, 3.0, and 5.0 µg/0.2 µl) on the number (A and C) and duration (B and D) of defensive immobility (A and B) and escape/avoidance (C and D) exhibited by prey submitted to the confrontation with rattlesnakes in a polygonal arena (unconditioned fear) and to the experimental context without the predator (conditioned fear). Data are presented as mean + S.E.M., $n = 7$, 7, 10, 10, 10, and 10 for nor-BNI at 5.0 µg/0.2 µl (No threat), Sal (No threat), Sal, nor-BNI at 1.0, 3.0, and 5.0 µg/0.2 µl (Threatened), respectively; $^{+++}$, $p < 0.001$ comparing Sal-no threat group and Sal-threatened group (according to student's *t*-test for independent samples); *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$ compared to respective Sal-treated group during the exposure of prey to the rattlesnakes (unconditioned fear) or the exposure of prey to the experimental context without the predator (conditioned fear), according to the one-way ANOVA, followed by Dunnett's *post hoc* test.

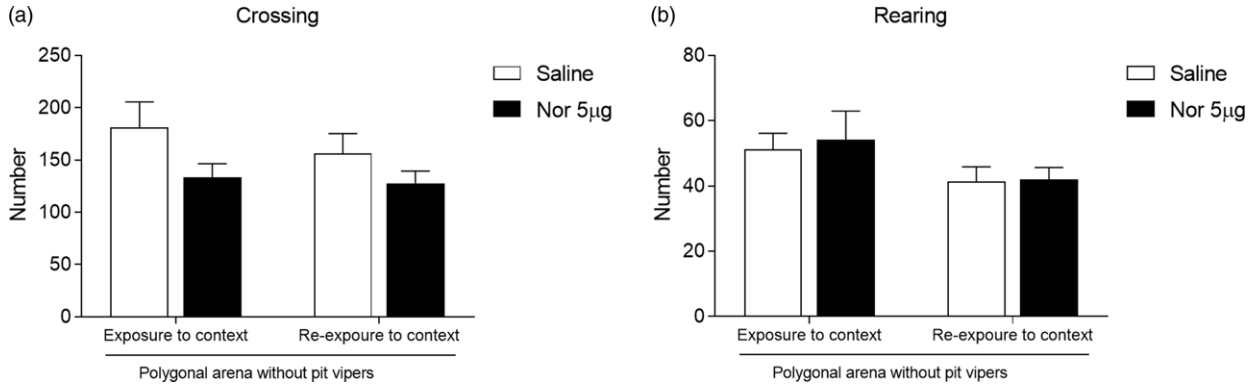

Figure 7. Effect of intracollicular pretreatment with physiological saline (Sal) or nor-binaltorphimine (nor-BNI) 5.0 µg/0.2 µl on the motor behaviour expressed by number of crossing (A) and rearing (B) exhibited by rats exposed and re-exposed to the experimental context (polygonal arena without rattlesnakes). Data are presented as the mean + S.E.M.; $p > 0.05$ in all comparisons, according to student's *t*-test for unpaired samples ($n = 7$ in each group).

The behavioural responses of rattlesnakes were similar to those displayed by pit vipers either confronted by mice (Almada *et al.*, 2022) or by rats (Calvo *et al.*, 2019b, c) as well as to defensive/offensive behaviour displayed by other Viperidae snakes confronted by male and female rats (Ferreira-Sgobbi *et al.*, 2022) and male mice (de Paula Rodrigues and Coimbra, 2022). The most expressive defensive/offensive behaviours of rattlesnakes confronted by specific germ free Wistar rats in the non-enriched polygonal arena for snakes panic test were either threatening posture with rattle movements and defensive posture, followed by exploratory behaviour, with low threatening attack (strikes without bite) and offensive attack (strikes with bite).

Although, depending of the Viperidae species of snakes (rattlesnakes and either *Bothrops jararaca* or *urutu-cruzeiro* lancehead pit vipers) we reported in literature discrimination of different ethologic parameters related to threatening and defensive postures displayed the predator (Almada *et al.*, 2022; de Paula Rodrigues and Coimbra, 2022; Ferreira-Sgobbi *et al.*, 2022), in this work we considered as threatening posture the elevation of head and the anterior third of the body. During that behavioural response, the rattlesnake moves the head following the movements of prey. Considering the defensive posture, we considered a retreated backward movement followed by immobility with body in a coil shape, as recently reported by Almada *et al* (2022). These both responses were more frequently displayed by rattlesnakes

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when they were in the presence of fearless prey. The ethologic sequence of those defensive responses displayed by the potential predator was threatening posture, with vigorous rattle movements, followed by either threatening or offensive strikes, and finally by defensive posture with the body in a coil shape with the head on the upper body ring. Despite the discrimination between these behavioural responses displayed by Viperidae snakes commonly reported in literature by our team (Calvo et al., 2019b, c; Almada *et al*., 2021; de Paula Rodrigues and Coimbra, 2022; de Paula Rodrigues and Coimbra, 2022), these both responses can be considered a defensive behaviour (Almada *et al*., 2022) of wild snakes, but displayed in different degrees of a threatening situation. Rattlesnakes display elevation of the first third of the body in a sigmoid shape with increased rattle sound to show how aversive and dangerous they are and display immobility with body in a coil shape when they are in a more silent and cautious behavioural performance. Both defensive and offensive strikes can be displayed in both situations at any moment. Rattlesnakes displayed an attentive behaviour during all prey versus predator confrontation.

The pretreatment of the central nucleus of the IC with nor-binaltorphimine at higher concentrations caused anxiolytic-like effect, significantly decreasing frequency and duration of defensive attention, and the incidence of flat back approach and startle, and a clear panicolytic-like effect, decreasing frequency and duration of defensive immobility and escape behaviour displayed by Wistar rats in the presence of rattlesnakes. The exposure of Wistar rats to the experimental context without the potential predator, performed 24 h after the intracollicular microinjections of nor-binaltorphimine most administered at higher concentrations, caused a significant decrease in frequency (3.0 and 5.0 μg), and duration (3.0 and 5.0 μg) of defensive attention, in duration of defensive immobility (3.0 and 5.0 μg), and in frequency (1.0 and 5.0 μg) and duration (all doses) of active avoidance. These findings suggest that the blockade of κ-opioid receptor in the central nucleus of the IC causes both anxiolytic- and panicolytic-like effect in a dangerous situation.

Despite the controversy regarding proaversive (Bals-Kubik *et al*., 1989) and antiaversive effects (Motta and Brandão, 1993; Nisbett *et al*., 2024; Kawaminami *et al*., 2024) of several opioid agonists and antagonists, paradoxical effects that seem to be related to either high or low dose, respectively of each opioid drug administered (da Silva *et al*., 2017), a consistent panicolytic-like effect of the pretreatment of the IC with a selective κ-opioid receptor antagonist was already reported by our team (Calvo *et al*., 2019a, b). Osaki *et al.* (2003) showed that microinjections of nor-binaltorphimine at different concentrations (2.5 and 5.0 μg/0.2 μl) in the IC significantly increased the escape behaviour thresholds elicited by electrical stimulations of central and pericentral nuclei of the IC. However, although Portoghese *et al.* (1987) demonstrated that both binaltorphimine and nor-binaltorphimine are highly potent and selective κ-opioid receptor antagonists, Birch *et al* (1987) showed evidence that in vivo nor-binaltorphimine was effective antagonist only at high doses and was not very selective between μ- and κ- opioid receptors, and its function as a potent κ-opioid receptor antagonist is not maintained in vivo.

On the other hand, Patkar *et al* (2013), demonstrated in binding experiments the physical presence of nor-binaltorphinime in mouse brain over 21 days after a single administration, suggesting its long-lasting antagonistic effect on κ-opioid receptor. This approach suggested physicochemical and pharmacological properties of nor-binaltorphimine contributing to the prolonged κ-opioid receptor-selective blockade. There is also evidence that the nor-binaltorphimine produces prolonged κ-opioid receptors inactivation by a c-Jun N-terminal kinase-based molecular mechanism (Bruchas *et al*., 2007; Reichard *et al*., 2020), and the long-lasting antagonistic effects of nor-binaltorphimine was also pharmacokinetically supported by another study (Kishioka *et al*., 2013).

Interestingly, the selective blockade of both μ_1- and κ-opioid receptors in central and pericentral nuclei of the IC with naloxonazine and nor-binaltorphimine at the highest concentration (5.0 μg/0.2 μl), and after either 24 h or 2 h, respectively, significantly decreased escape behaviour panic-like reactions expressed by running and jumps elicited by intracollicular blockade of $GABA_A$ receptor with microinjections of the selective antagonist bicuculline in a concentration of 40 ng/ 200 nl (Calvo *et al*., 2019a). However, opposite effect of μ- and κ-opioid signalling mechanisms in the dPAG on anxiety-like behaviour displayed by rats in the elevated plus-maze test was also reported (Nobre *et al*., 2000). However, in the IC of rats the blockade of both μ- and κ-opioid receptor with either peripheral or central administrations of naloxonazine (Coimbra *et al*., 2000; Calvo *et al*., 2019a), and intracollicular microinjections of nor-binaltorphimine (Osaki *et al*., 2003; Calvo *et al*., 2019a) cause panicolytic-like effect.

In conclusion, microinjections of nor-binaltorphimine at higher concentrations in IC of rats threatened by wild rattlesnakes in a dangerous environment causes anxiolytic- and panicolytic-like effects. These findings suggest that the decrease in κ-opioid receptor signalling in the caudal midbrain tectum significantly decreases panic attacks. These data reinforce the propositions of our team of medical use of opioid antagonists as coadjutant medicines for the treatment of panic syndrome.

Availability of data. The datasets generated or analysed during the current study are available from the corresponding author upon reasonable request.

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Competing interests. The authors declare that they have no conflicts of interest with respect to the work presented herein.

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