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Neostriatum neuronal $TRPV_1$-signalling mediates striatal anandamide at high concentration facilitatory influence on neostriato-nigral dishinhibitory GABAergic connections

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ABSTRACT

Rationale: Several lines of evidence have demonstrated that the cannabinoid type 1 receptor (CB_1) is found in the caudate nucleus and putamen (CPu) in addition to the substantia nigra pars reticulata (SNpr). Here, we investigated the role of endocannabinoid neuromodulation of striato-nigral disinhibitory projections on the activity of nigro-collicular GABAergic pathways that control the expression of unconditioned fear-related behavioural responses elicited by microinjections of the $GABA_A$ receptor selective antagonist bicuculline (BIC) in the deep layers of the superior colliculus (dlSC).

Methods: Fluorescent neural tract tracers were deposited in either CPu or in SNpr. Wistar rats received injection of vehicle, anandamide (AEA), either at low (50 pmol) or high (100 pmol) concentrations in CPu followed by bicuculline microinjections in dlSC.

Results: Connections between CPu, the SNpr and dlSC were demonstrated. The $GABA_A$ receptor blockade in dlSC elicited panic-like behaviour. AEA at the lowest concentration caused a panicolytic-like effect that was antagonised by the CPu pretreatment with AM251 at 100 pmol. AEA at the highest concentration caused a panicogenic-like effect that was antagonised by the CPu pretreatment with 6-iodonordihydrocapsaicin (6-I-CPS) at different concentrations (0.6, 6, 60 nmol).

Conclusion: These findings suggest that while pre-synaptic CB1-signalling subserves an indirect facilitatory effect of AEA on striato-nigral pathways causing panicolytic-like responses through midbrain tectum enhanced activity, post-synaptic $TRPV_1$-signalling in CPu mediates AEA direct activation of striato-nigral disinhibitory pathways resulting in increasing dlSC neurons activity and a panicogenic-like response. All these actions seem to depend on the interface with the nigro-collicular inhibitory GABAergic pathways.

1. Introduction

It is known that the hypothalamus, the amygdaloid complex, and the midbrain tectum structures, such as the periaqueductal grey matter (PAG), the intermediate (ilSC) and deep (dlSC) layers of the superior colliculus, in addition to the inferior colliculus (IC) comprise a fight and flight system or the encephalic aversion system (EAS), an important neural network for the organisation of unconditioned and conditioned fear-related behavioural responses (Anseloni et al., 1999; Borelli et al., 2004; Brandão et al., 1994, 2005; Adams, 2006). The PAG has been considered a mesencephalic output of EAS activity, coordinating the generation and elaboration of innate fear (Graeff, 1981) alongside other structures, such as the dlSC (Coimbra and Brandão, 1993; da Silva et al., 2013) and IC (Castellan-Baldan et al., 2006). It has been demonstrated that either the administration of excitatory amino acids or the microinjection of bicuculline in both these mesencephalic and diencephalic

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structures induce defensive reactions in rodents (Brandão et al., 1982; Brandão et al., 1999; Freitas et al., 2009; de Freitas et al., 2013a; b). However, the escape responses elicited by medial hypothalamus is interspersed by exploratory behaviour and is oriented to protected areas, while the escape behaviour elicited by electrical or chemical stimulation of PAG and corpora quadrigemina is non-oriented (Ullah et al., 2015). The cerebral cortex and some hypothalamic nuclei are indirectly and directly connected to the dorsal midbrain (Castellan-Baldan et al., 2006; Falconi-Sobrinho et al., 2017; Ullah et al., 2015). The substantia nigra, pars reticulata (SNpr), is a critical neural interface for connections between the nucleus caudatus-putamen (CPu) and the continuum comprised by dlSC and PAG (Castellan-Baldan et al., 2006; Almada et al., 2018). Both the SNpr (Coimbra et al., 2017b; Almada et al., 2015, 2021) and medial hypothalamus nuclei, such as the dorsomedial hypothalamus (dos Anjos-Garcia and Coimbra, 2019), are rich in the cannabinoid type 1 (CB_1) receptor. There is evidence for CPu-pallidum-SNpr disinhibitory and SNpr-midbrain tectum inhibitory GABAergic projections modulating defensive behaviour elaborated by PAG and corpora quadrigemina neural substrates (Castellan-Baldan et al., 2006; da Silva et al., 2018). These connections showed to be modulated by endocannabinoids, considering that microinjections of anandamide in the SNpr decreased panic-like reactions displayed by mice threatened by venomous snakes in the polygonal arena for snake panic test (Almada et al., 2021). Indeed, facing dangerous environments with potential predators prey elicit defensive attention (Lobão-Soares et al., 2008; Coimbra et al., 2017a,c), defensive immobility (dos Anjos-Garcia and Coimbra, 2020; de Paula et al., 2022), escape to safe places (Almada and Coimbra, 2015; Almada et al., 2021, 2022), and inhibitory avoidance (de Paula Rodrigues and Coimbra, 2022), and treatment of SNpr with anandamide attenuates escape responses elicited by $GABA_A$ receptors blockade in the dlSC in a dangerous environment (Almada et al., 2021).

Anandamide, one of the main endocannabinoids, is synthesised upon demand, and acts as retrograde messenger on CB_1 and CB_2 receptors (Alger and Kim, 2011). The endogenous cannabinoid anandamide also activates the transient receptor potential cation channel subfamily V member 1 (TRPV1) in both laboratory animals and humans (Smart et al., 2000; dos Anjos-Garcia et al., 2017; dos Anjos-Garcia and Coimbra, 2019). Indeed, there is evidence that anandamide was identified as an agonist for the recombinant human TRPV1 by screening a large array of bioactive substances using a FLIPR-based calcium assay and electrophysiology (Smart et al., 2000). Whereas the activation of the CB_1 receptor diminishes defensive behavioural responses, the activation of the $TRPV_1$ channel has been shown to be anxiogenic (Aguiar et al., 2015). Microinjections of $TRPV_1$ agonists in the PFC (Rubino et al., 2008) and in the PAG (Mascarenhas et al., 2013) produce anxiogenic responses. Indeed, endocannabinoids generally produce biphasic effects on defensive responses and are anxiogenic or ineffective at higher doses, likely because of the interaction with $TRPV_1$ receptors (Moreira et al., 2012).

The hypothesis of the present study is that endocannabinoids can participate as neuromodulators, acting in the control of the elaboration of defense, evoked by aversive stimuli, and organised by dlSC. It is also possible that endogenous cannabinoids recruit CB_1 receptors and $TRPV_1$ endovaniloid channels in the neostriatum during the modulation of defensive responses evoked by intramesencephalic microinjection of a $GABA_A$ receptor antagonist. Therefore, it is possible that blocking such receptors in the neostriatum will impair the effect of anandamide on midbrain neurons activity. The hypothesis of the present study is that endovanilloids can also participate as neuromodulators in the neostriatum. This neuromodulation, acting on neostriato-nigral disinhibitory GABAergic projections might modify the activity of nigrocollicular GABAergic inhibitory pathways that modulate unconditioned fear-induced defensive responses elaborated by dlSC neurons.

2. Material and methods

2.1. Animals

Male Wistar rats (*Rattus norvergicus*; Rodentia; Muridae) aged 9–11 weeks (weighing 200–250 g, n = 8 per group) at the beginning of the experiment, from the animal facility of the School of Medicine of Ribeirão Preto of the University of São Paulo (FMRP-USP), were used. These animals were housed four in a group in Plexiglas cages and provided free access to food and water throughout the experiment. They were kept in the experimental room for 48 h prior to the experiment and were maintained on a 12 h light/12 h dark cycle (lights on at 7:00 a.m.) at 23–25 °C. The study protocols complied with the Ethical Commission in Animal Experimentation of the FMRP-USP recommendations, which fulfils the principles of ethics for animal research adopted by the National Council for Control of Animal Experimentation (CONCEA) and were approved by the Commission of Ethics in Animal Research (FMRP-USP CEUA process 0074/2015).

2.2. Stereotactic surgery

The animals were anaesthetised with 92 mg/kg ketamine and 9.2 mg/kg xylazine, submitted to a stereotaxic surgery as previously described (da Silva et al., 2018) for unilateral implantation of two stainless steel guide cannulae (0.6 mm outside diameter, 0.4 mm inside diameter) in the midbrain aimed at the dlSC and in the telencephalon, aiming the CPu, according to the following coordinates (with Bregma as the reference), dlSC (anterior/posterior (AP), − 6.36 mm; medial/lateral (ML), ± 1.2 mm; dorsal/ventral (DV), 3.6 mm) and CPu (AP, − 1.08 mm; ML, ± 3.8 mm; DV, 4.8 mm). The cannulae were fixed to the skull with acrylic resin and two stainless-steel screws. Each guide cannula was sealed with stainless steel wire to protect it from blockage. Each rat was treated with an intramuscular injection of penicillin G-benzathine (120, 000 UI; 0.2 ML) followed by intramuscular injection of the analgesic and anti-inflammatory drug flunixin meglumine (2.5 mg/kg). Afterwards, the rats were allowed 5 days to recover from the surgical procedure.

2.3. Neuroanatomical tractography

A bi-directional neural tract tracer (AlexaFluor Texas red-conjugated dextran; 3000 MW; Molecular Probes, Eugene, OR, USA) was microinjected either into the CPu or in the SNpr in independent groups of animals in a volume of 0.5 μL over the course of 5 min. Infusions were delivered using an infusion pump (Stoelting, Kiel, Wisconsin, USA) through a polyethylene tube (PE10) attached to a dental needle. The dental needle was left in place for 2 min after the end of each microinjection to allow local drug diffusion. After completing the microinjection procedure, the dental needle was removed, and the skin was sutured. Then, 7 days after the neural tract tracer microinjections, mice were deeply anaesthetised with intramuscular injections of 92 mg/kg ketamine (Ketamina®) and 9.2 mg/kg xylazine (Dopaser®) and perfused intracardially with physiological saline followed by 4 % paraformaldehyde (PFA, Sigma) dissolved in 0.1 M phosphate buffer (pH 7.4). The encephalon was removed, post-fixed in PFA for 4 h, and then transferred to 10% and 20% sucrose dissolved in 0.1 M sodium phosphate buffer, pH 7.3, at 4 °C for at least 12 h in each solution. The nervous tissue was immersed in 2-methylbutane (Sigma), frozen in dry ice (30 s), embedded in Tissue-Tek, sectioned (20 mm thick) using a cryostat (CM 1950, Leica, Wetzlar, Germany) at 20 °C and mounted with Fluoromount with DAPI (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) on silanised slides. After this procedure, the forebrain slices with the bi-directional neural tract tracer were mounted on glass slides and cover-slipped with mineral oil, and both the SNpr and CPu were observed under fluorescence microscopy (AxioImager Z1 with APOTOME II, Zeiss, Oberkochen, Germany).

2.4. Drugs and injections

The GABA-A receptor selective antagonist bicuculline (BIC; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline (0.9 % NaCl) and centrally administered in a dose of 40 ng (Almada and Coimbra, 2015; da Silva et al., 2018). Anandamide (AEA; Tocris, Bristol, UK) was dissolved in Tocrisolve TM 100 (a solvent that contains a 1:4 ratio of soy oil/water, emulsified with the block co-polymer Pluronic F68) and used at 50 and 100 pmol (da Silva et al., 2020). The transient receptor potential vanilloid type 1 ($TRPV_1$) agonist 6-iodonordihydrocapsaicin (6-I-CPS) was dissolved in vehicle (10 % dimethyl sulfoxide; DMSO) (dos Anjos-Garcia et al., 2017; Medeiros et al., 2020). The CB_1 receptor antagonist AM251 (Tocris Bioscience, Bristol, UK) at 100 pmol was diluted in 10% DMSO (Almeida-Santos et al., 2013; dos Anjos-Garcia et al., 2017). A needle (outer diameter, 0.3 mm) 1 mm longer than the guide cannula, attached by a polyethylene tube (PE-10) to a 5 μL syringe (Hamilton, Reno, Nevada, USA), was introduced through the guide cannula and used to deliver the drugs into the CPu and dlSC using an infusion pump (Stoelting, Wood Dale, IL, USA). Each vehicle and the drug were injected in a volume of 200 nL into each encephalic structure reached in the present investigation. The rats received ipsilateral injections of vehicle or AEA in the CPu and, 5 min after this procedure, either vehicle or BIC was intramesencephalically (dlSC) microinjected. Immediately after the last injection, either the exploratory (control) or the defensive behaviour of rats was recorded in a circular arena.

2.5. Behavioural procedures

Five days after the surgery to implant the guide cannula in the dlSC and CPu, the rats were habituated in a circular enclosure for 10 min. After habituation, the rats were gently wrapped in a cloth and held to receive the pharmacological treatments in the CPu and dlSC. The $GABA_A$ receptor blockade in the dlSC was performed 5 min after each CPu pretreatment (physiological saline, AEA, AM251, or 6-I-CPS). Immediately after each treatment in the dlSC, each rodent was placed in a circular arena (a circular enclosure, 60 cm in diameter and 50 cm high) situated in an experimental compartment illuminated with seven dichroic lamps (390 lx at the arena floor level). The intensity of the illumination was recorded with a digital luxmeter (MLM 1332 Minipa Luxímetro, Minipa do Brazil, Joinville, SC, Brazil). The frequency of the following responses was recorded for subsequent ethological analysis: behavioural defensive reactions, expressed as the number of behavioural events and duration of defensive alertness (alertness; a response operationally defined as the interruption of an ongoing behaviour and immobility up to 5 s, which is characterised by an attentive posture with small head movements, rearing, and smelling of the surrounding air); the number of behavioural events and duration of defensive immobility (a panic attack-like response of freezing; operationally defined as immobility at least for 6 s followed by two or more of the following autonomic reactions: defecation, urination, piloerection, or exophthalmos), and the number of behavioural events and duration of escape behaviour (a panic attack-like behaviour) expressed by running without orientation and/or jumping (elevation of the four paws from the floor of the open field). All these behavioural responses were recorded by a video camera (Sony Handycam HDR-CX350, Konan, Minato-ku, Tokyo, Japan) for 15 min immediately after the microinjection of BIC into the dlSC. Each animal received a maximum of two treatments. All experiments were performed from 7:00 a.m. to 2:00 p.m. The researchers were blind to each pharmacological treatment.

2.6. Histology

Upon completion of the experiments, the animals were anaesthetised with 92 mg/kg ketamine and 9.2 mg/kg xylazine (Dopaser) and perfused through the left cardiac ventricle, and the brain/midbrain slices were obtained as previously described (da Silva et al., 2017, 2018). The positions of the guide cannula tip and the injector needle tract were identified using a motorised photomicroscope (AxioImager Z1, Zeiss, Oberkochen, Germany) and displayed on modified diagrams from Paxinos and Watson's rat brain in stereotaxic coordinates atlas (2006). Data from rats with guide cannula located outside the dlSC or CPu were not included in the statistical analysis, but they are presented in Fig. 8 to show the specificity of brain site stimulation and/or drug infusion.

2.7. Statistics

Psychopharmacological data are represented as the mean ± standard error of the mean (S.E.M.) and were analysed by the GraphPad Prism 8 software (GraphPad Inc.). Normality and homogeneity in the sampled distributions were confirmed with the Shapiro-Wilk's test of normality and Bartlett`s test of homogeneity of variances. Based on the results of these tests, appropriate parametric tests, two-tailed unpaired *t*-test, one and two-way ANOVAs were performed. In the two-way ANOVA, the factors were first and second microinjections into the CPu and the Tukey's post-hoc test was used to detect significant differences. $P < 0.05$ was considered statistically significant.

3. Results

A schematic distribution of each histologically confirmed site of microinjection of drugs in the telencephalic (CPu) and mesencephalic (dlSC) structures is shown in Fig. 1 (neuroanatomical experiments) and in Fig. 3 (psychopharmacological experiments). Here, we studied the striato-nigral disinhibitory/nigro-tectal inhibitory γ-aminobutyric acid (GABA)ergic pathways control of dorsal midbrain neurons activity during the elaboration of panic attack-like behaviour, and the involvement of endocannabinoid mechanisms in the CPu on the expression of defensive behaviours of rats elicited by GABAergic disinhibition of the dlSC neurons.

3.1. Experiment 1: Neuroanatomical investigation of striato-nigral and nigro-tectal neural connexions

Cerebral microinjections of the bi-directional neural tract tracer in CPu (Fig. 1A-D) showed neural tract tracer-labelled perikaya in the internal pyramidal layer of the primary motor cortex (data not shown), neural tract tracer-labelled fibres and terminal buttons from substantia nigra, pars compacta neurons connected to CPu (Fig. 2A; right inferior corner), profuse neural tract tracer-labelled output projections from CPu (Fig. 2B), descending through the ipsilateral base of the cerebral peduncle (Fig. 2C; left inferior corner), and neural tract tracer-labelled perikarya situated in the SNpc (Fig. 2C, right superior corner). Profuse CPu outputs significantly invade the reticulate division of the substantia nigra (Fig. 2D), in which neural tract tracer-labelled appositions suggesting synaptic contacts were found surrounding perikarya in SNpr (Fig. 2E). Ventral midbrain deposits of the bi-directional neural tract tracer, targeting the SNpr (Fig. 1E-H), showed neural tract tracer-labelled neuronal perikarya situated in the CPu (Fig. 2A, on the left), perikarya, axons and terminal buttons situated in the dorsomedial columns of the periaqueductal grey matter (Fig. 2F), dorsolateral columns of the periaqueductal grey matter (Fig. 2G), axons and terminal buttons situated in the dlSC (Fig. 2H), perikarya, axons and terminal buttons situated in the intermediated layers of the superior colliculus (ilSC), as shown in Fig. 2I, and neural tract tracer-labelled perikarya situated in the internal pyramidal layer of the medial rostral cerebral frontal lobe cortex prelimbic division (data not shown) Fig. 3.

3.2. Experiment 2: CB1-signalling panicolytic-like effect of AEA at lower doses in CPu

This investigation was performed aiming to determine the role of CPu CB_1 receptor in panicolytic consequences of intra-CPu

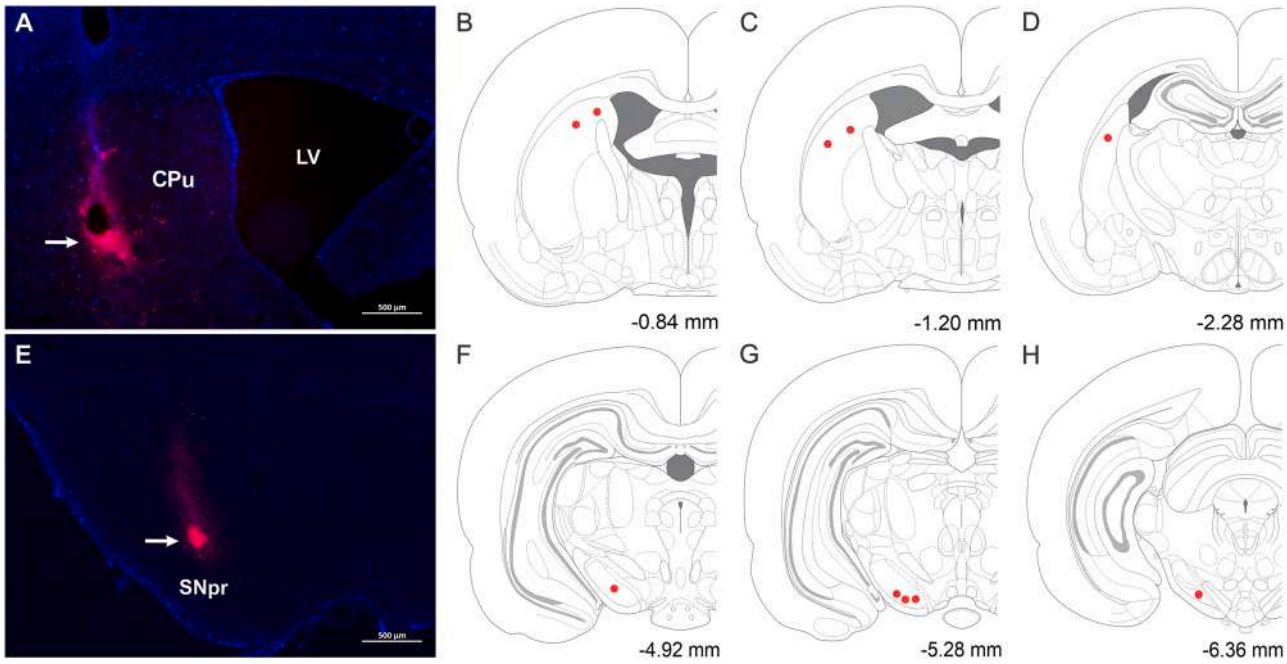


Fig. 1. A: Fotomicrograph of a coronal section of *Rattus norvegicus* telencephalon showing a representative site of microinjection of AlexaFluor-Texas red-conjugated dextran neural tract tracer (AFTrD) in the nucleus caudatus-putamen (CPu). B: Fotomicrograph of a transverse section of *R. norvegicus* midbrain showing a representative site of microinjection of AFTrD in the substantia nigra pars reticulata (SNpr). B-D and F-H: Schematic transverse sections of *R. norvegicus* telencephalon (B-D) and mesencephalon (F-H) showing histologically confirmed injection sites (red closed circles) of AFTrD in the neural tissue. LV: Lateral ventricle.

microinjections of AEA at lower doses.

According to the one-way analysis of variance, there was a statistically significant effect of the CPu treatment with AEA at 50 pmol on the number [$F(4, 35) = 115.1$; $p < 0.001$] and duration [$F(4, 35) = 12.5$; $p < 0.001$] of the defensive alertness elicited by $GABA_A$ receptor blockade in dlSC (Fig. 4A and B). Indeed, the pretreatment of CPu with AEA in a dose of 50 pmol caused a significant decreased in number (Tukey's post hoc test: $p < 0.001$) and duration (Tukey's post hoc test: $p < 0.01$) of defensive alertness, when compared to the treatment of the CPu with vehicle followed by BIC administration in the dlSC (Fig. 4A and B). The pretreatment of CPu with AM251 in a dose of 100 pmol + AEA in a dose of 50 pmol significantly minimised the anxiolytic-like effect of AEA (Tukey's post hoc test: $p < 0.001$), considering the incidence of defensive attention when compared to the treatment of the CPu with vehicle + AEA (50 pmol) followed by BIC administration in the dlSC (Fig. 4A). The pretreatment of CPu with AEA in a dose of 50 pmol alone caused any intrinsic effect neither in number nor in duration of defensive attention ($p > 0.05$ in both cases) as compared to the control group [vehicle + vehicle (CPu) + vehicle (dlSC)], as shown in Fig. 4A and B.

Regarding the defensive immobility, the $GABA_A$ receptor antagonism in the dlSC caused a statistically significant increase in the number (Tukey's post hoc test: $p < 0.001$) and the duration (Tukey's post hoc test: $p < 0.001$) of the defensive immobility behaviour (Fig. 4C and D). According to a one-way ANOVA, there was a statistically significant effect of the treatment on the number [$F(4, 35) = 56.94$; $p < 0.001$] and duration [$F(4, 35) = 15.08$; $p < 0.001$] of defensive immobility. Treatment of CPu with AEA caused a statistically significant decrease in the incidence (Tukey's post hoc test: $p < 0.001$) and duration (Tukey's post hoc test: $p < 0.001$) of the defensive immobility when compared to the treatment of CPu with vehicle followed by BIC administration in the dlSC (Fig. 4C and D). Pretreatment of CPu with AM251 in the dose of 100 pmol caused a significant impairment in the panicolytic-like effect of AEA on incidence and duration of defensive immobility (Tukey's post hoc test: $p < 0.001$ in both cases) elicited by $GABA_A$ receptor blockade in dlSC, as shown in Fig. 4C and D. Treatment of CPu with AM251 (100 pmol) + AEA (50 pmol) caused a statistically significant increase in the incidence (Tukey's post hoc test: $p < 0.001$) of defensive immobility when compared to the treatment of CPu with vehicle followed by BIC administration in the dlSC (Fig. 4C). The pretreatment of CPu with AEA in a dose of 50 pmol alone caused any intrinsic effect neither in number nor in duration of defensive immobility ($p > 0.05$ in both cases) as compared to the control group [vehicle + vehicle (CPu) + vehicle (dlSC)], as shown in Fig. 4C and D.

The $GABA_A$ receptor blockade in dlSC elicited escape behaviour expressed by running and jumps as shown in Fig. 5. Considering the escape behaviour expressed by running, according to a one-way ANOVA, there was a statistically significant effect of the treatment on incidence [$F(4, 35) = 51.64$; $p < 0.001$] and duration [$F(4, 35) = 19.17$; $p < 0.001$] of that panic attack-like response. Treatment of CPu with AEA in a dose of 50 pmol caused a statistically significant decrease in the incidence (Tukey's post hoc test: $p < 0.001$) and the duration (Tukey's post hoc test: $p < 0.001$) of running, when compared to the treatment of CPu with vehicle followed by BIC administration in the dlSC (Fig. 5A and B). The blockade of CB_1 receptors in CPu with AM251 at 100 pmol caused a statistically significant impairment of the panicolytic-like effect of AEA at 50 pmol on the incidence of escape behaviour expressed by running (Tukey's post hoc test: $p < 0.001$) and caused a trend towards an increase in the duration of running in comparison to the effect of AEA (Fig. 5 B).

Regarding the escape response expressed by jumping, according to a one-way ANOVA, there were statistically significant effects of the treatment on the incidence [$F(4, 35) = 29.44$; $p < 0.001$] of jumping. The pretreatment of the CPu with AEA caused a panicolytic-like effect, significantly decreasing the incidence of jumping (Tukey's post hoc test: $p < 0.01$) in comparison with rats that received vehicle into the CPu and BIC in the dlSC (Fig. 5 C). The escape behaviour expressed by running and jumping elicited by $GABA_A$ receptor blockade in dlSC was followed by a significant enhancement of crossings [$F(4,35) = 58.56$; $p < 0.001$; Tukey's post hoc test: $p < 0.001$] (Fig. 5D) that decreased significantly ($p < 0.001$) after the CPu pretreatment with AEA in a dose of 50 pmol. Pretreatment of CPu with AM251 (100 pmol) caused a trend towards an increase of both incidence of escape behaviour expressed by jumping

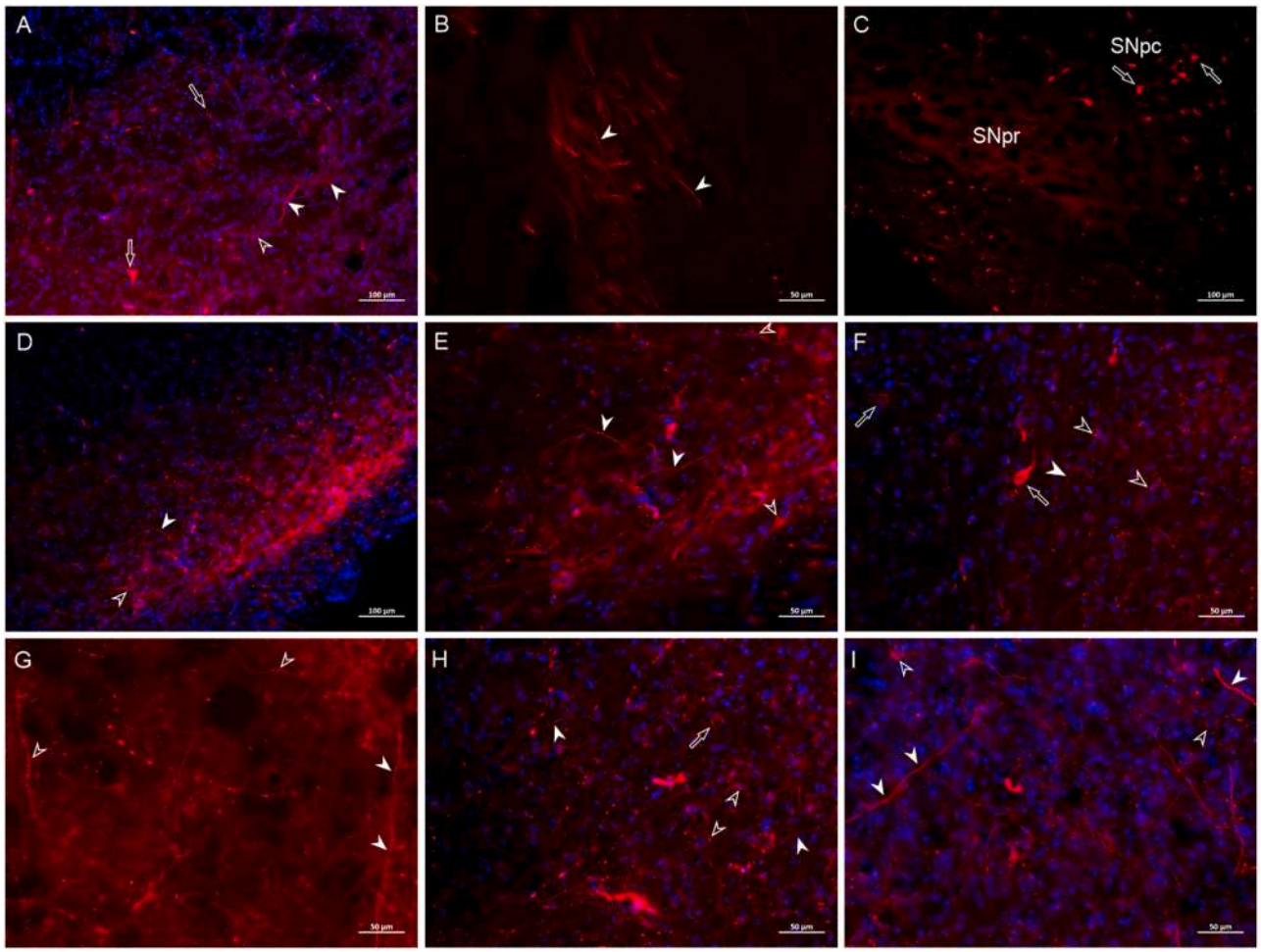


Fig. 2. Photomicrographs of coronal sections of the Wistar rats telencephalon (A and B) and transverse section of ventral (C-E) and dorsal (F-I) midbrain, showing AlexaFluor-Texas red-conjugated dextran neural tract tracer-labelled perikarya (open arrows), neuronal fibres (closed arrowheads) and appositions suggesting synaptic contacts (open arrowheads) situated in the nucleus caudatus-putamen (A and B) substantia nigra (C-E), dorsomedial columns of the periaqueductal grey matter (F), dorsolateral columns of the periaqueductal grey matter (G), deep layers of the superior colliculus (H), and in the intermediate layers of the superior colliculus (I).

and in number of crossings elicited by microinjection of BIC in the dlSC (Fig. 5 C and D).

The pretreatment of CPu with AEA in a dose of 50 pmol alone caused any intrinsic effect neither in number nor in duration of escape behaviour expressed by running and jumping ($p > 0.05$ in all cases) as compared to the control group [vehicle + vehicle (CPu) + vehicle (dlSC)], as shown in Fig. 5.

3.3. *Experiment 3: TRPV1-signalling panicogenic-like effect of AEA at high doses in CPu*

Microinjections of vehicle into the CPu followed by pharmacological blockade of $GABA_A$ receptors in dlSC elicited unconditioned fear-related defensive behaviours, such as defensive attention/alertness, defensive immobility (freezing) and escape, expressed by running and jumping, when compared with rats that received vehicle in both the CPu and dlSC (Figs. 6 and 7).

Considering the alertness, according to the one-way ANOVA, there was a statistically significant effect of the treatment on the number [$F(6, 42) = 23.87$; $p < 0.001$] and duration [$F(6, 42) = 18.33$; $p < 0.001$] of the defensive attention. The $GABA_A$ receptor blockade in dlSC significantly enhanced the incidence and duration of defensive attention (Fig. 6 A and B). The CPu pretreatment with AEA at the highest dose (100 pmol) caused a significant enhancement of the duration of defensive attention (Tukey's post hoc test: $p < 0.001$). The pretreatment of CPu with the selective $TRPV_1$ vanilloid receptor antagonist 6-I-CPS at the highest dose caused a significant decrease in the incidence of defensive attention as shown in Fig. 6 A (Tukey's post hoc test: $p < 0.001$). The anxiogenic-like effect of AEA microinjected in CPU at the higher dose (100 pmol), increasing the duration of alertness, was significantly impaired by CPu pretreatment with 6-I-CPS at 0.6, 6, and 60 nmol (Tukey's post hoc test: $p < 0.001$ in all cases), as shown in Fig. 6B.

Regarding the freezing response, according to a one-way ANOVA, there was a statistically significant effect of the treatment on the number [$F(6, 42) = 17.06$; $p < 0.001$] and duration [$F(6, 42) = 23.21$; $p < 0.001$] of defensive immobility. The $GABA_A$ receptor antagonism in the dlSC caused a statistically significant increase in the number (Tukey's post hoc test: $p < 0.001$) and the duration (Tukey's post hoc test: $p < 0.05$) of the defensive immobility behaviour (Fig. 6 C and D). Treatment of CPu with AEA at a higher dose (100 pmol) caused a trend towards an increase in the incidence of defensive immobility (Fig. 6 C), and a statistically significant increase in the duration of the defensive immobility (Tukey's post hoc test: $p < 0.001$) when compared to the treatment of CPu with vehicle followed by BIC administration in the dlSC (Fig. 6 C and D). The pretreatment of CPu with 6-I-CPS in the doses of 0.6, 6, 60 nmol + AEA caused a significant decreased in number (Tukey's post hoc test: $p < 0.05$, $p < 0.001$, $p < 0.001$) and duration

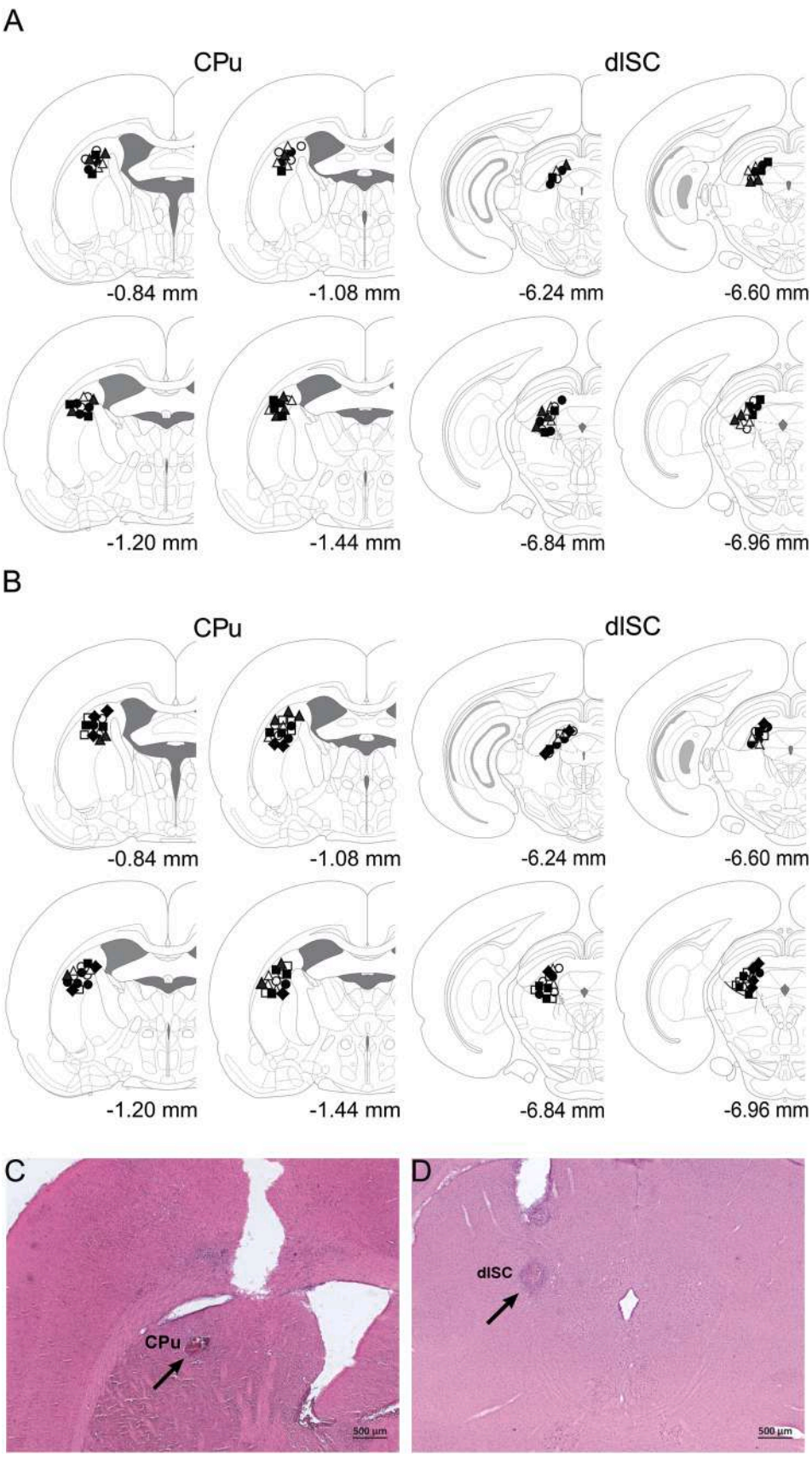


Fig. 3. A: Schematic coronal sections of the *Rattus norvegicus* telencephalon (A and B on the left) and mesencephalon (A and B on the right) showing histologically confirmed injection sites of microinjections of drugs in the neostriatum (CPu) and deep layers of the superior colliculus (dlSC) as follow: (A) vehicle + vehicle (CPu)-vehicle (dlSC) (○), vehicle + vehicle (CPu)-bicuculline (●), vehicle + AEA/50 pmol (CPu)-bicuculline (dlSC) (▲), vehicle + AEA/50 pmol (CPu)-vehicle (dlSC) (Δ), AM251/100 + AEA/50 pmol (CPu)-bicuculline (dlSC) (■); (B) vehicle + vehicle (CPu)-vehicle (dlSC) (○), vehicle + vehicle (CPu)-bicuculline (●), vehicle + AEA/100 pmol (CPu)-bicuculline (dlSC) (▲), 6-I-CPS/0.6 nmol + AEA/100 pmol (CPu)-bicuculline (dlSC) (Δ), 6-I-CPS/6 nmol + AEA/100 pmol (CPu)-bicuculline (dlSC) (◆), 6-I-CPS/60 nmol + AEA/100 pmol (CPu)-bicuculline (dlSC) (■), 6-I-CPS/60 nmol + vehicle (CPu)-bicuculline (dlSC) (□) depicted in modified diagrams from Paxinos and Watson's rat brain in stereotaxic coordinates atlas (2006). The number of points in the figure is fewer than the total number of rats because of overlapping injection sites. C-D: Photomicrographs of transverse sections of telencephalon (C) and mesencephalon (D) showing (black arrows) representative sites of microinjection of the drugs in the CPu and in the dlSC.

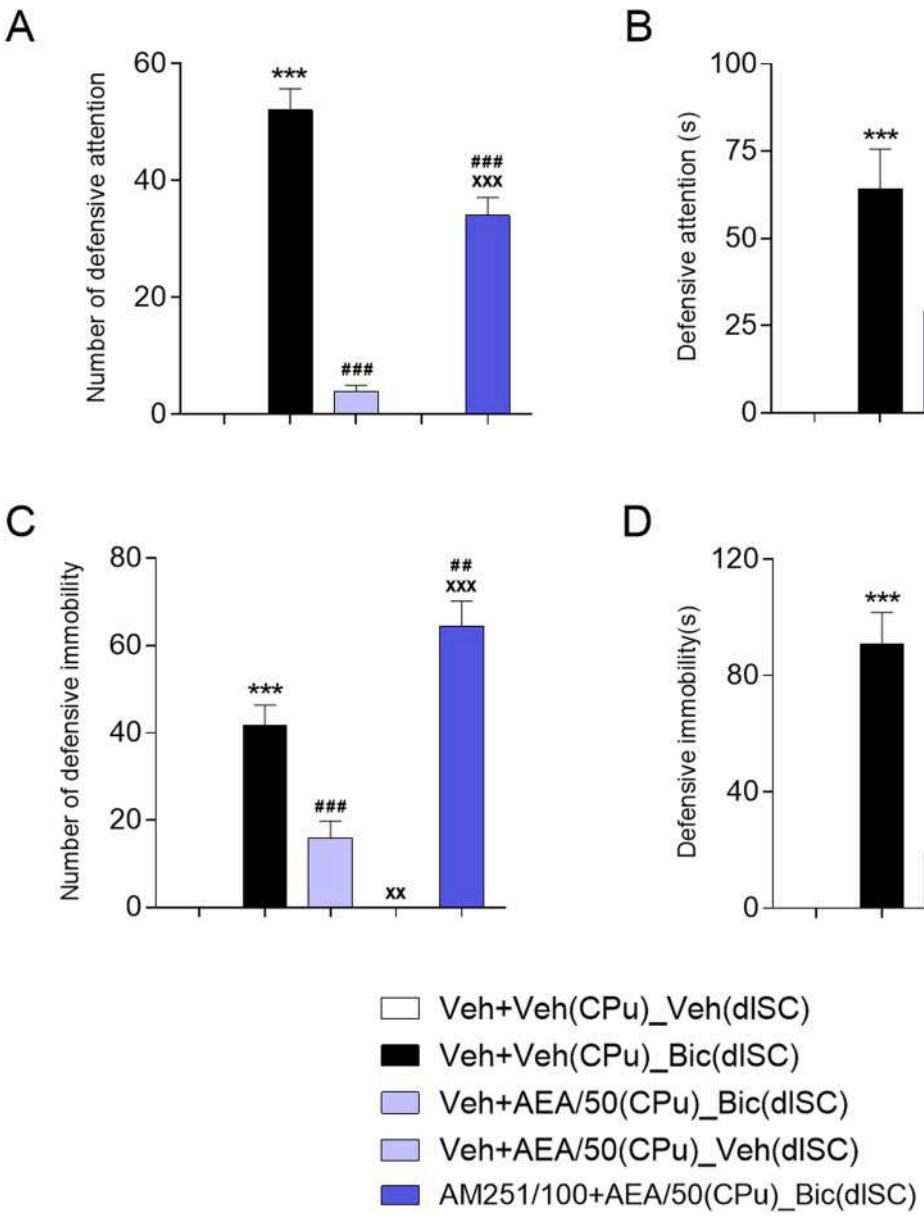


Fig. 4. Effect of pretreatment of the neostriatum (CPu) with vehicle, anandamide (AEA) 50 pmol or AM251 100 pmol (n = 8) on defensive behaviour expressed as the number and duration of defensive alertness (A and B) and defensive immobility (C and D), elicited by $GABA_A$ receptors blockade with microinjection of bicuculline at 40 ng into the deep layers of the superior colliculus (dlSC). The columns represent the mean and the bars represent the standard error of the mean; * ** $p < 0.001$, compared with the vehicle (CPu) + vehicle (dlSC)-treated group; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, compared with the vehicle (CPu) + bicuculline (dlSC)-treated group, ^{xx} $p < 0.01$, ^{xxx} $p < 0.01$ compared with the vehicle + AEA at 50 pmol (CPu) + bicuculline (dlSC)- treated group according to the one-way ANOVA followed by Tukey's post hoc test.

(Tukey's post hoc test: $p < 0.001$ in all cases) of defensive immobility when compared to the treatment of the CPu with vehicle followed by BIC administration in the dlSC. Treatment of CPu with 6-I-CPS in a dose of 60 nmol + AEA (100 pmol) caused a statistically significant decrease in the incidence of the defensive immobility (Tukey's post hoc test: $p < 0.01$) when compared to the treatment of CPu with 6-I-CPS at the lowest dose (0.6 nmol) plus AEA (100 pmol), followed by BIC administration in the dlSC (Fig. 6 C). Treatment of CPu with 6-I-CPS in the dose of 60 nmol + vehicle caused a statistically significant increase in the incidence of the defensive immobility (Tukey's post hoc test: $p < 0.01$) when compared to the treatment of CPu with 6-I-CPS in the dose of 60 nmol + AEA (100 pmol) followed by BIC administration in the dlSC (Fig. 6 C).

BIC microinjections in the dlSC elicited a panic attack-like escape behaviour expressed by running, as shown in Fig. 7 A and B. According to a one-way ANOVA, there was a statistically significant effect of the treatment on the incidence [$F(6, 42) = 32.09$; $p < 0.001$] and duration [$F(6, 42) = 19.17$; $p < 0.001$] of running. The $GABA_A$ receptor antagonism in the dlSC caused a statistically significant increase in the number (Tukey's post hoc test: $p < 0.001$) and the duration (Tukey's post hoc test: $p < 0.01$) of that panic attack-like response (Fig. 7A and B). Treatment of CPu with AEA at higher dose (100 pmol) followed by a $GABA_A$ receptor blockade in dlSC caused a statistically significant increase in both the incidence (Tukey's post hoc test: $p < 0.01$) and the duration (Tukey's post hoc test: $p < 0.01$) of running, when compared to the treatment of CPu with vehicle followed by BIC administration in the dlSC (Fig. 7A and B). The pretreatment of CPu with 6-I-CPS at 0.6, 6, 60 nmol + AEA (100 pmol) caused a significant impairment in the panicogenic-like effect of AEA at the higher dose of 100 pmol, decreasing both the incidence (Tukey's post hoc test: $p < 0.01$,

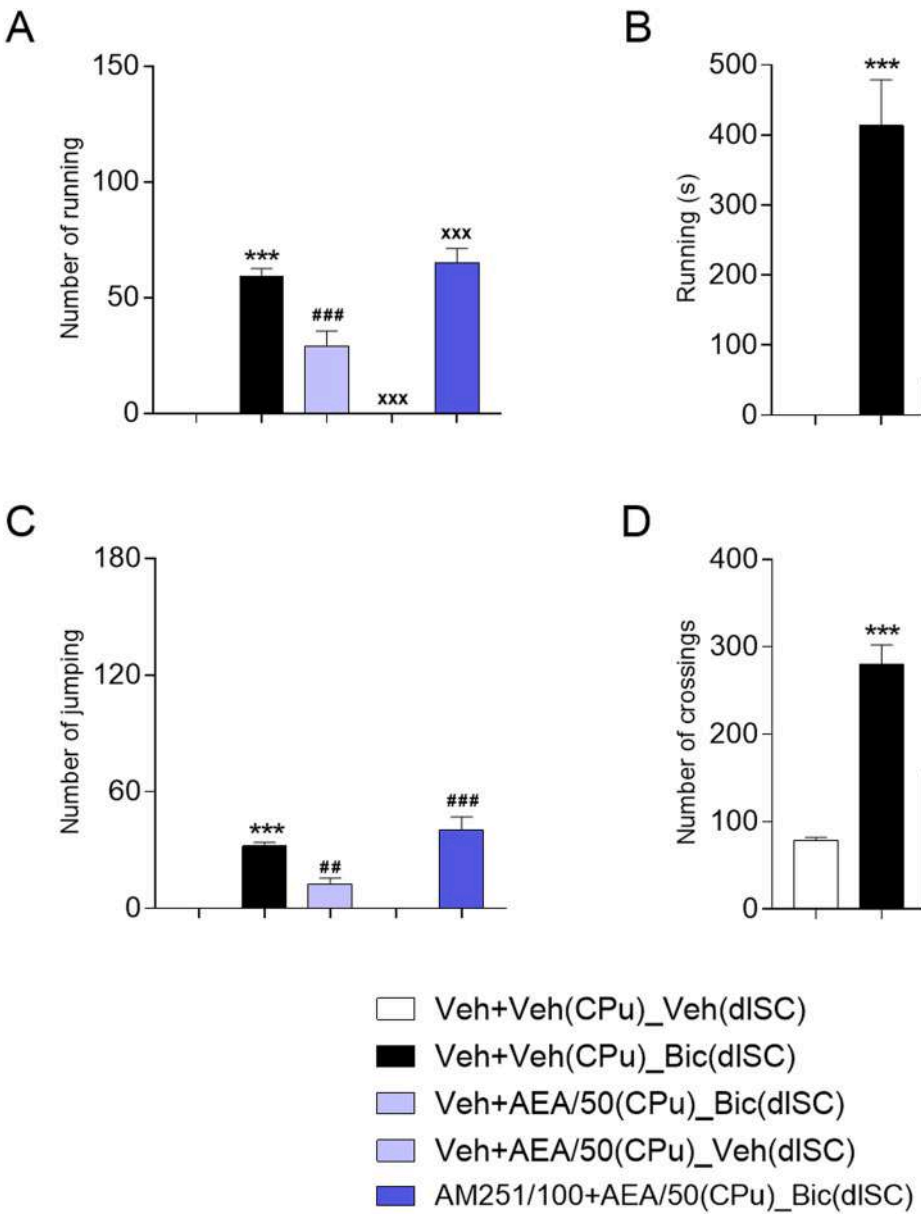


Fig. 5. Effect of pretreatment of the neostriatum (CPu) with vehicle, anandamide (AEA) 50 pmol or AM251 100 pmol (n = 8) on defensive behaviour expressed as the number and duration of escape behaviour by running (A and B) and jumping (C and D), elicited by $GABA_A$ receptors blockade with injection of bicuculline in the deep layers of the superior colliculus (dlSC). The columns represent the mean and the bars represent the standard error of the mean; * ** $p < 0.001$, compared with the vehicle (CPu) + vehicle (dlSC)-treated group; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, compared with the vehicle (CPu) + bicuculline (dlSC)-treated group, ^{xxx} $p < 0.001$ compared with the vehicle + AEA at 50 pmol (CPu) + bicuculline (dlSC)- treated group according to the one-way ANOVA followed by Tukey's post hoc test.

$p < 0.001$, $p < 0.001$, respectively) and duration (Tukey's post hoc test: $p < 0.001$ in all cases) of escape behaviour expressed by running, compared to the treatment of the CPu with vehicle followed by BIC administration in the dlSC (Fig. 7A and B). Treatment of CPu with 6-I-CPS in the dose of 60 nmol + vehicle caused a statistically significant increase in the incidence (Tukey's post hoc test: $p < 0.01$) and duration (Tukey's post hoc test: $p < 0.01$) of running, as compared to the treatment of CPu with 6-I-CPS in the dose of 60 nmol + AEA (100 pmol), followed by BIC administration in the dlSC (Fig. 7A and B).

The blockade of $GABA_A$ receptors in the dlSC also elicited an escape behaviour expressed by jumping, as shown in Fig. 7 C. According to a one-way ANOVA, there were statistically significant effects of the treatment on the incidence [$F(6, 42) = 84.72$; $p < 0.001$] of jumping escape behaviour. Treatment of the CPu with AEA at higher dose (100 pmol) caused a panicogenic-like effect, significantly increasing the incidence of jumping (Tukey's post hoc test: $p < 0.001$) in comparison with rats that received vehicle into the CPu and BIC in the dlSC (Fig. 7 C). Pretreatment of the CPu with 6-I-CPS at different doses (0.6, 6, 60 nmol) caused a significant impairment in the panicogenic-like effect of AEA at higher dose, significantly decreasing the incidence (Tukey's post hoc test: $p < 0.001$) of escape behaviour expressed by jumping evoked by microinjection of BIC in the dlSC, as shown in Fig. 7C. Treatment of CPu with 6-I-CPS in the dose of 60 nmol + vehicle caused a statistically significant increase in the incidence (Tukey's post hoc test: $p < 0.001$) of jumping when compared to the treatment of CPu with 6-I-CPS in the dose of 60 nmol + AEA (100 pmol), followed by BIC administration in the dlSC (Fig. 7C).

Regarding the locomotor activity of crossing, according to a one-way ANOVA there was a significant effect of the treatment [$F(6,42) = 276,8$; $p < 0.01$]. The escape behaviour elicited by microinjections of bicuculline in the dlSC increased the number of crossing, that was also high after the treatment of the CPu with AEA at higher dose (100 pmol)

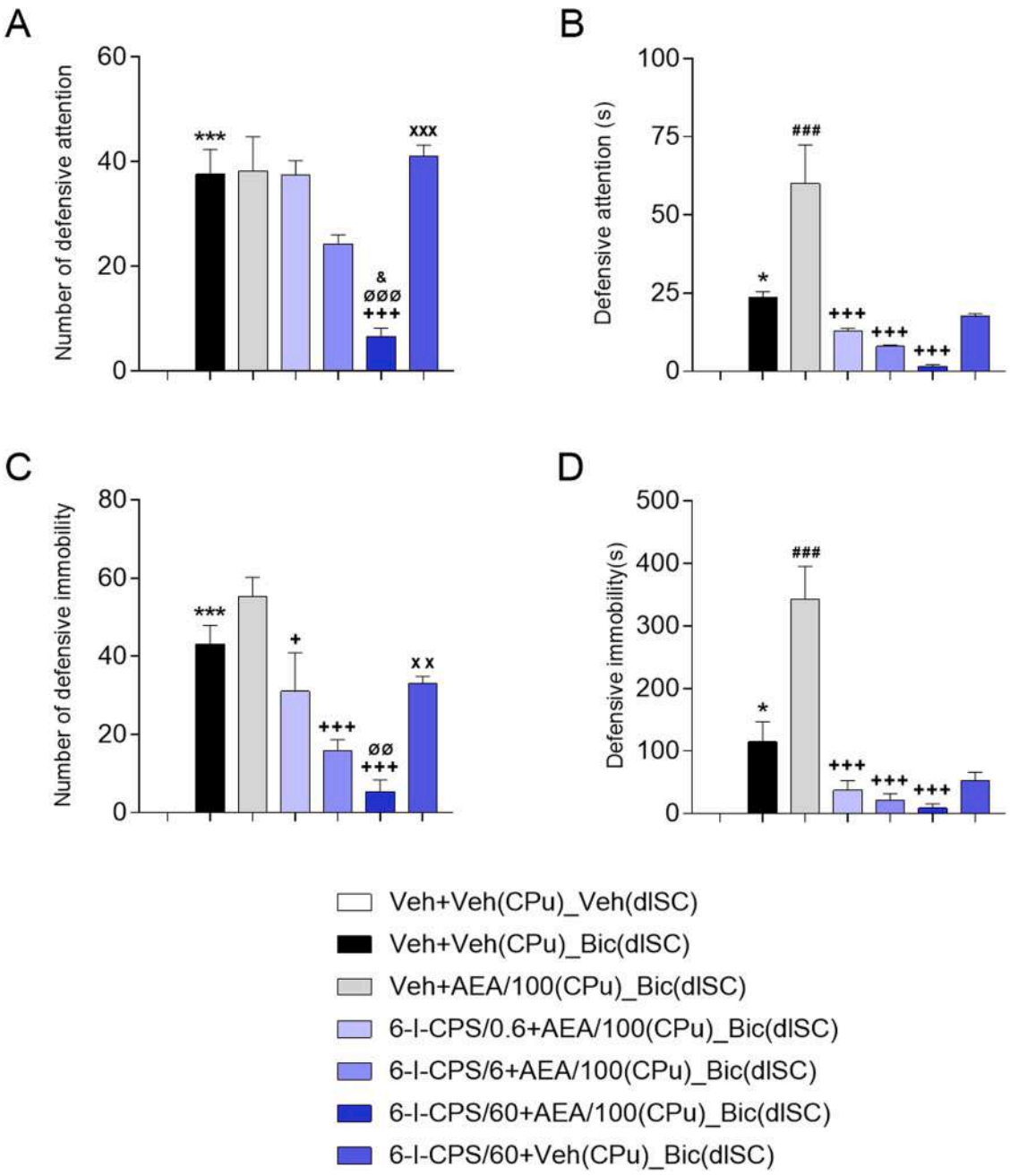


Fig. 6. Effect of pretreatment of the neostriatum (CPu) with vehicle, anandamide (AEA) 100 pmol or 6-I-CPS at 0.6, 6 and 60 nmol (n = 8) on defensive behaviour expressed as the number and duration of defensive alertness (A and B) and defensive immobility (C and D), elicited by GABA A receptors blockade with microinjection of bicuculline at 40 ng in the deep layers of the superior colliculus (dlSC). The columns represent the mean and the bars represent the standard error of the mean; * $p < 0.05$, * ** $p < 0.001$, compared with the vehicle (CPu) + vehicle (dlSC)-treated group; $^{###}$ $p < 0.001$, compared with the vehicle (CPu) + bicuculline (dlSC)-treated group, $^{+}$ $p < 0.05$, $^{+++}$ $p < 0.001$, compared with the AEA at 100 pmol (CPu) + bicuculline (dlSC)-treated group, ^{ØØ} $p < 0.01$, ^{ØØØ} $p < 0.001$, compared with the 6-I-CPS at 0.6 nmol + AEA at 100 pmol (CPu) + bicuculline (dlSC)-treated group, $^{&}$ $p < 0.05$, compared with the 6-I-CPS at 6 nmol + AEA at 100 pmol (CPu) + bicuculline (dlSC)-treated group, ^{xx} $p < 0.01$, ^{xxx} $p < 0.001$ compared with the 6-I-CPS at 60 nmol + AEA at 100 pmol (CPu) + bicuculline (dlSC)-treated group according to the one-way ANOVA followed by Tukey's post hoc test.

(Tukey's post hoc test; $p < 0.01$); a panicogenic like effect impaired by CPu pretreatment with 6-I-CPS at different doses (Tukey's post hoc test; $p < 0.001$ in all cases), as shown in Fig. 7D. Treatment of CPu with 6-I-CPS in the dose of 60 nmol + vehicle caused a statistically significant increase in the incidence (Tukey's post hoc test: $p < 0.001$) of crossings when compared to the treatment of CPu with 6-I-CPS in the dose of 60 nmol + AEA (100 pmol), followed by BIC administration in the dlSC (Fig. 7D).

Neither microinjections of BIC, cannabinoid nor vanilloid drugs outside the CPu and dlSC caused/influenced panic attack-like defensive immobility and escape (running and jumping responses) behaviours, as shown in Fig. 8.

4. Discussion

The neuroanatomical experiments demonstrated that CPu neurons send projection to the ventral midbrain through the crus cerebri basis, reaching the reticulate division of the substantia nigra, and receive axon terminals from SNpc neurons, as already demonstrated in Literature (Matias et al., 2019). Profuse axonal fibres from CPu neurons surround

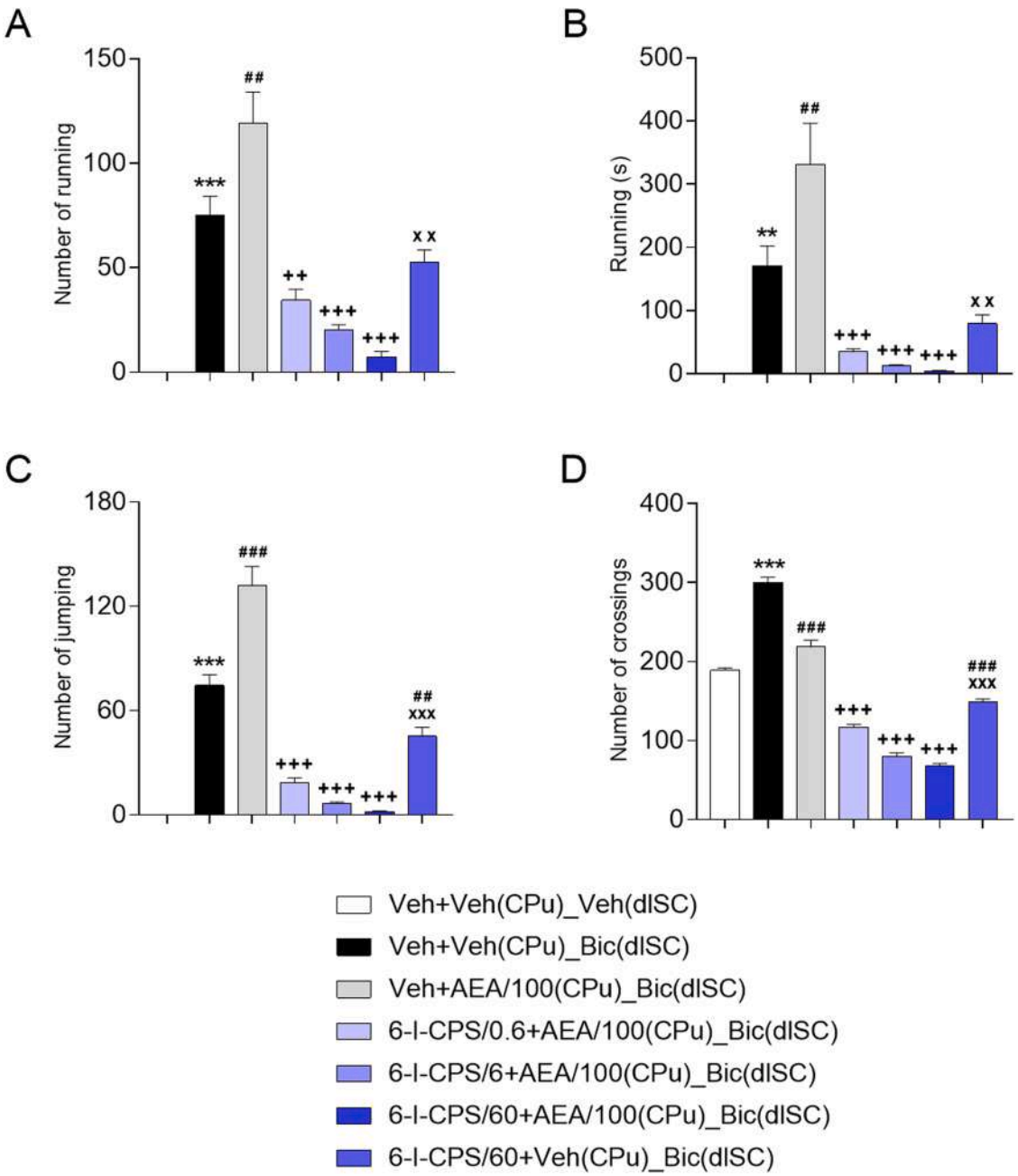
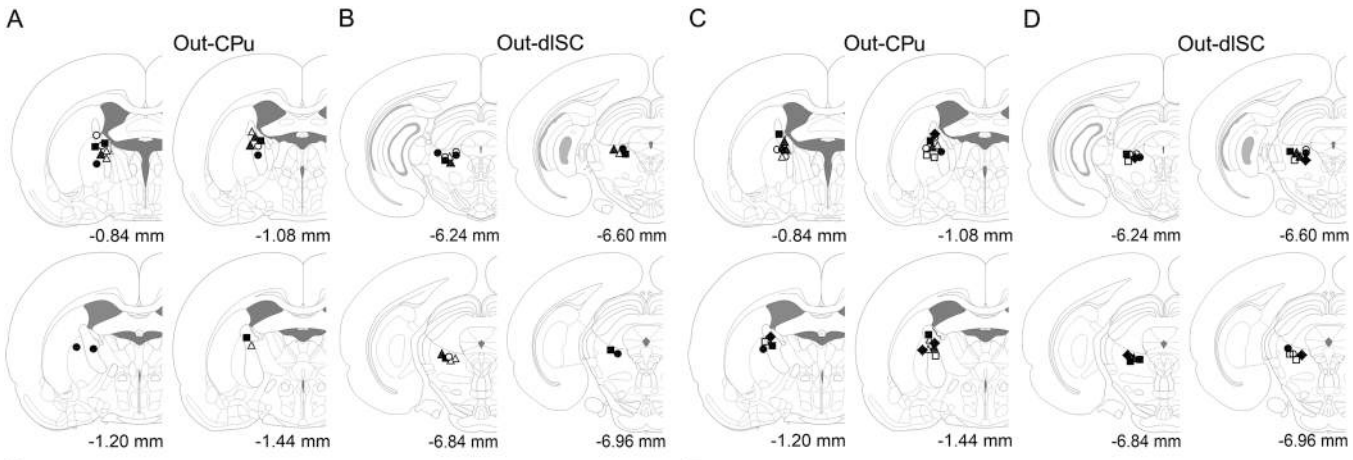


Fig. 7. Effect of pretreatment of the neostriatum (CPu) with vehicle, anandamide (AEA) at 100 pmol or 6-I-CPS at 0.6, 6 and 60 nmol (n = 8) on defensive behaviour expressed as the number and duration of escape behaviour by running (A and B) and jumping (C and D), elicited by $GABA_A$ receptors blockade with injection of bicuculline in the deep layers of the superior colliculus (dlSC). The columns represent the mean and the bars represent the standard error of the mean; * * $p < 0.01$, * ** $p < 0.001$, compared with the vehicle (CPu) + vehicle (dlSC)-treated group; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, compared with the vehicle (CPu) + bicuculline (dlSC)-treated group, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$, compared with the AEA at 100 pmol (CPu) + bicuculline (dlSC)-treated group, ^{xx} $p < 0.01$, ^{xxx} $p < 0.001$, compared with the 6-I-CPS at 60 nmol + AEA at 100 pmol (CPu) + bicuculline (dlSC)-treated group according to the one-way ANOVA followed by Tukey's post hoc test.

SNpr neurons with appositions suggesting synaptic contacts. The SNpr neurons send projections to dmPAG, dlPAG, dlSC, and ilSC. Some of these connections are reciprocated, such those between SNpr and both dmPAG, dlSC, and ilSC, as already reported by our team (Eichenberger et al., 2002; Ribeiro et al., 2005), comprising the intramesencephalon tecto-nigral opioid modulatory links. Reciprocated connections between the ilSC and the SNpc were also already retorted in Literature (Comoli et al., 2003; Ribeiro et al., 2005) and seem to be related to the control of movements of the eyes and head to visual salient stimuli, for example during predatory behaviour (Comoli et al., 2003). However, in our work we addressed the CPu-SNpr disinhibitory pathways controlling the activity of SNpr-midbrain tectum GABAergic inhibitory projections that exert a control of unconditioned fear-related behaviour organised by dorsal midbrain neurons either electrically or chemically stimulated (Coimbra and Brandão, 1993; Ribeiro et al., 2005; Castellan-Baldan et al., 2006) or also activated during prey versus predator confrontations (Almada et al., 2021).

GABAergic disinhibition in the dorsal midbrain, performed with microinjections of a $GABA_A$ receptor antagonist (bicuculline) in the dlSC, induced behavioural defense responses, characterised mainly by alertness, defensive immobility, and non-oriented escape behaviours. These panic attack-like reactions seem to result from the decrease of GABAergic neurotransmission in the dorsal midbrain and consequently momentary interruption of the tonic inhibitory control exerted by GABA



E

Defensive behaviours	Experimental out groups					
	Veh+Veh(CPu)+ Veh(dlSC)	Veh+Veh(CPu)+ Bic(dlSC)	Veh+AEA50(CPu)+ Bic(dlSC)	Veh+AEA50(CPu)+ Veh(dlSC)	AM251/100+AEA50(CPu) +Bic(dlSC)	Statistic
Nº defensive attention	-	1;1.5;2	0;0;1	0;0;0.75	0;0;1.5	$H_{(4)}$ = 8.55; p>0.05
Defensive attention (s)	-	0.59;0.62;0.66	0;0;0.3	0;0;0.45	0;0;0.48	$H_{(4)}$ = 9.34; p>0.05
Nº defensive immobility	-	-	-	-	-	-
Defensive immobility (s)	-	-	-	-	-	-
Nº running	-	-	-	-	-	-
Running (s)	-	-	-	-	-	-
Nº jumping	-	-	-	-	-	-
Nº crossings	32;39;41	65;78.5;90.5	51;63;86	54.5;59.5;66.75	69.25;93;101.8*	$H_{(4)}$ = 11.8; p<0.05

F

Defensive behaviours	Experimental out groups								
	Veh+Veh(CPu)+ Bic(dlSC)	Veh+Veh(CPu)+ Bic(dlSC)	Veh+AEA100(CPu)+ Bic(dlSC)	6-I-CPS/0.6+ AEA/100(CPu)+ Bic(dlSC)	6-I-CPS/6+ AEA/100(CPu)+ Bic(dlSC)	6-I-CPS/60+ AEA/100(CPu)+ Bic(dlSC)	6-I-CPS/60+ Veh(CPu)+ Bic(dlSC)	Statistic
Nº defensive attention	-	0.25;1.5;2	0;1;2	0;0.5;075	0.25;1.5;2	0;0.5;2.5	0;0.5;1.75	$H_{(6)}$ = 5.48; p>0.05
Defensive attention (s)	-	0.05;0.60;1.08	0;0.41;1.98	0;0.50;1.91	0.10;0.55;1	0;0.20;1.11	0;0.15;0.84	$H_{(6)}$ = 5.06; p>0.05
Nº defensive immobility	-	-	-	-	-	-	-	-
Defensive immobility (s)	-	-	-	-	-	-	-	-
Nº running	-	-	-	-	-	-	-	-
Running (s)	-	-	-	-	-	-	-	-
Nº jumping	-	-	-	-	-	-	-	-
Nº crossings	23;29.5;33	15.7;27;42.7	21;29;30	22;38;39	13.5;17;20.5	17.2;19.5;21	30.5;38;42.5	$H_{(6)}$=12.5; p>0.05

Fig. 8. Top: Schematic coronal sections of the *Rattus norvegicus* telencephalon (B and C) and transverse sections of the mesencephalon (B and D) showing histologically confirmed sites of microinjections of the following drugs outside the caudate nucleus-putamen (CPu) and deep layers of the superior colliculus (dlSC): vehicle + vehicle (CPu)-vehicle (dlSC) (○) (n = 3); vehicle + vehicle (CPu)-bicuculline (dlSC) (●) (n = 4); vehicle + AEA/50 (CPu)-bicuculline (dlSC) (▲) (n = 3); vehicle + AEA/50 pmol (CPu)-vehicle (dlSC) (Δ) (n = 4); AM251/100 pmol + AEA/50 pmol (CPu)-bicuculline (dlSC) (■) (n = 4) (A and B), and vehicle + vehicle (CPu)-vehicle (dlSC) (○) (n = 4); vehicle + vehicle (CPu)-bicuculline (dlSC) (●) (n = 4); vehicle + AEA/100 pmol (CPu)-bicuculline (dlSC) (▲) (n = 3); 6-I-CPS/0.6 nmol + AEA/100 pmol (CPu)-bicuculline (dlSC) (Δ) (n = 4); 6-I-CPS/6 nmol + AEA/100 pmol (CPu)-bicuculline (dlSC) (◆) (n = 4); 6-I-CPS/60 nmol + AEA/100 pmol (CPu)-bicuculline (dlSC) (■) (n = 4); 6-I-CPS/60 nmol + veh (CPu)-bicuculline (dlSC) (□) (n = 4) (C and D) depicted in modified drawings from the rat brain in stereotaxic coordinates atlas by Paxinos and Watson's (2006). The number of points in the figure is fewer than the total number of rats because of overlapping injection sites. Bottom: Lack of effect of each treatment in CPu and dlSC on unconditioned fear-related behaviour (E and F).

on the neural substrates that process aversive stimuli in the corpora quadrigemina and in the PAG, the main output from the encephalic aversion system (Coimbra and Brandão, 1993; Coimbra et al., 2006) activated in threatening situations such as those in which prey are confronted by wild snakes (Paschoalin-Maurin et al., 2018; de Paula et al., 2022).

Previous researchers have found that striatonigral GABAergic pathways exert a strong inhibitory influence on neurons of the SNpr (Fallon and Laughlin, 1985; Bolam et al., 2000; Ribeiro et al., 2005). These disinhibitory inputs affects the activity of nigrotectal projection neurons, which are also inhibitory (Chevalier et al., 1981; Coimbra et al., 1989; Coimbra and Brandão, 1993; Eichenberger et al., 2002; Castellan-Baldan et al., 2006). It has been postulated that GABAergic striatonigral pathways recruit GABA receptors in SNpr modulating nigrotectal GABAergic inhibitory connections that culminate in the modulation of defensive responses related to panic attacks either induced by electrical and chemical stimulations of the dorsal midbrain (Ribeiro et al., 2005; Castellan- Baldan et al., 2006) or in more naturalistic aversive panic models, such as those based on prey versus serpents confrontation paradigms (Almada and Coimbra, 2015; Almada et al., 2021, 2022).

Aiming to clarify the role of neostriatal cannabinoid system in the control of the striatonigral/ nigrocollicular GABAergic pathways, a pretreatment of the neostriatum with anandamide at different doses was performed. The defensive behaviours induced by the administration of bicuculline in the dlSC were reduced by the previous administration of anandamide at a dose of 50 pmol in the neostriatum. That cannabinoid receptors agonist at low dose was able to significantly reduce most of these defensive behaviours. These findings corroborate a previous report from our team (da Silva et al., 2020). However, here we presented new evidence regarding the cannabinoid receptor recruited by AEA. Indeed, the anxiolytic- and panicolytic-like effects caused by administration of AEA in the nucleus caudatus-putamen are dependent of the recruitment of in situ CB_1 cannabinoid receptor, considering that the pretreatment of the neostriatum with the CB_1-receptor antagonist AM251 in a dose of 100 pmol caused an impairment in the antiaversive effect of anandamide on the defensive attention (alertness), defensive immobility (freezing) and escape responses elicited by $GABA_A$ receptor blockade in the dlSC with intracollicular microinjections of bicuculline.

Similar antiaversive effects caused by intracerebral treatment with anandamide was recently demonstrated in medial hypothalamus (dos Anjos-Garcia et al., 2017). Furthermore, recent evidence suggests that manipulation of cannabinoid neurotransmission may have effects on the acquisition and expression of contextual fear conditioning. Studies have shown that reduced activation of CB_1 receptors leads to a decrease in conditioned fear expression, whereas CB_1 agonist administration caused an increase in fear-related behaviour expression (Haller et al., 2002). In addition, another study suggested that administration of AM251 also decreased the acquisition of contextual fear (Arenos et al., 2006).

Interestingly, we demonstrated that the endocannabinoid neuromodulator anandamide is able of altering the defensive responses elaborated by dlSC neurons when administered in the nucleus caudatus-putamen. To explain that effect we might consider that AEA acts on CB_1 receptors situated on presynaptic axonal terminals of cortico-striatal glutamatergic projections, decreasing the activity of that excitatory connections, therefore resulting in a decreased activity of the GABAergic striatonigral disinhibitory pathways, and the SNpr neurons will control the activity of dlSC and dorsal periaqueductal grey matter (dPAG) neurons where at least part of the defensive response will be organised.

Regarding the dlPAG, there is evidence that endocannabinoids can modulate defensive behaviour through CB_1 receptors in animals subjected to the elevated maze test, an animal model of anxiety. In fact, part

of the anxiolytic-like effect induced by anandamide seems to be signalled by the CB_1 receptor, since the anandamide produced anxiolytic effect was prevented by intramesencephalic pretreatment with AM251 (Moreira et al., 2007). Similar CB1-signalled anandamide effect was found in the present work, highlighting the neostriatum. In this way, anandamide can modulate the defense behaviour organised by structures of the dorsal midbrain, acting either directly on the tectum or through the control of activity and the neostriatonigral/nigrocollicular pathways, as presently demonstrated.

Interestingly, the pretreatment of the CPu with AEA at the higher dose of 100 pmol caused a proaversive effect, significantly enhancing the duration of defensive attention, and defensive immobility, the incidence and duration of escape behaviour expressed by running, and the number of jumping.

Other authors have proposed that endogenous cannabinoid ligands, such as anandamide, may exert either panicolytic or panicogenic effects via activation of either CB_1 or $TRPV_1$ receptors, respectively (Casarotto et al., 2012). Indeed, high doses of this cannabinoid agonist have been reported to be anxiogenic, unlike low doses, which have been reported to be anxiolytic (Chhatwal and Ressler, 2007). This fact is possibly due probably to the activation of $TRPV_1$ and CB_1 receptors, respectively. In line with this view, some studies have shown that $TRPV_1$ antagonists have antiaversive effects, possibly because they allow endogenous anandamide to bind directly to CB_1 receptors. The same is true if we block the panicolytic effect of capsazepine ($TRPV_1$ antagonist) through pretreatment with CB_1 antagonists (Almeida-Santos et al., 2013).

To test the hypothesis that AEA at higher dose could recruit $TRPV_1$ vanilloid receptors in CPu, causing anxiogenic- and panicogenic-like responses, we pretreated the rats with the selective $TRPV_1$ receptor antagonist 6-I-CPS at different doses. That intrastriatal pretreatment with 6-I-CPS at any dose used in this work (0.6, 6, and 60 nmol) was able to reverse the facilitatory effect of anandamide on the responses of defensive attention, defensive immobility, and escape, evoked by the treatment of dlSC with bicuculline, suggesting that anandamide in a dose of 100 pmol exerts its proaversive effect by acting on $TRPV_1$ endovanilloid receptors.

In conclusion, the pretreatment of the CPu with AEA at low dose caused a CB_1-signalling anxiolytic- (decreasing both defensive attention incidence and duration) and panicolytic- (decreasing both incidence and duration of defensive immobility and escape behaviours) like effects on defensive behaviour elicited by $GABA_A$ receptor blockade in dlSC. On the other hand, the pretreatment of CPu with AEA at the highest dose caused a $TRPV_1$-signalling anxiogenic- (increasing the duration of defensive attention) and panicogenic- (increasing the duration of defensive immobility and both incidence and duration of escape behaviours) like effects on panic-like reactions elicited by dlSC GABAergic disinhibition.

These findings suggest that on-demand released endocannabinoids in the neostriatum can act either on presynaptic CB_1-cannabinoid receptors or on postsynaptic $TRPV_1$-vanilloid receptors, modulating either the corticostriatal excitatory inputs or the striatonigral disinhibitory outputs, with an indirect of direct influence on inhibitory nigrocollicular GABAergic pathways, therefore controlling the unconditioned fear-related emotional behaviour.

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CRediT authorship contribution statement

J.A. da Silva performed the psychopharmacological experiments, analysed data and wrote the manuscript; J.A. da Silva, G.R. Pigatto, P.M. Hernandes, and N.C. Coimbra performed neuroanatomical experiments; L.L. Falconi-Sobrinho performed statistical analyses and graphs; R.C. Almada and N.C.Coimbra interpreted data and wrote the manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest concerning the presented work.

Data Availability

Data will be made available on request.

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