European Neuropsychopharmacology (2019) 29, 858–870

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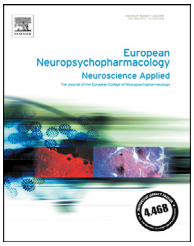

Dorsal raphe nucleus 5-Hydroxytryptamine 2A receptors are critical for the organisation of panic attack-like defensive behaviour and unconditioned fear-induced antinociception elicited by the chemical stimulation of superior colliculus neurons

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Received 14 December 2018; received in revised form 4 April 2019; accepted 29 May 2019

KEYWORDS
Defensive behaviour;
Panic disorder;
Fear-induced antinociception;
5-HT_{2A} receptor;
Deep layers of the superior colliculus

Abstract
Microinjections of N-methyl-D-aspartic acid (NMDA) in the midbrain tectum structures produce panic attack-like defensive behaviours, followed by an antinociceptive response. It has been suggested that fear-related defensive responses organised by brainstem neurons can be modulated by 5-hydroxytryptamine (5-HT). However, there is a shortage of studies showing the role of dorsal raphe nucleus (DRN) 5-HT_{2A} receptors in the modulation of panic-like behaviour and fear-induced antinociception organised by the superior colliculus (SC). The purpose of this study was to investigate the participation of DRN 5-HT_{2A} receptors in the modulation of panic attack-

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https://doi.org/10.1016/j.euroneuro.2019.05.007

like behaviour and antinociception evoked by intra-SC injections of NMDA. In experiment I, the animals received microinjections of physiological saline or NMDA (6, 9 and 12 nmol) in the deep layers of the SC (dlSC). In experiment II, the most effective dose of NMDA (12 nmol) or vehicle was preceded by microinjections of vehicle or the 5-HT$_{2A}$ receptor selective antagonist R-96544 at different concentrations (0.5, 5 and 10 nM) in the DRN. Both proaversive and antinociceptive effects elicited by intra-dlSC injections of NMDA were attenuated by DRN pretreatment with R-96544. In addition, a morphological analysis showed that 5-HT$_{2A}$ receptors are present in GABAergic interneurons in the DRN. Taken together, these findings suggest that DRN 5-HT$_{2A}$ receptors are critical for the modulation of both panic attack-like defensive behaviour organised by SC neurons and unconditioned fear-induced antinociception. A possible interaction between serotonergic inputs, GABAergic interneurons and serotonergic outputs from the DRN was also considered.

1. Introduction

Panic disorder is considered a subtype of the anxiety disorder spectrum, and it is characterised by unexpected and recurrent episodes of intense fear accompanied by physiological alterations, such as tachycardia, hyperventilation and increased blood pressure (DSM-V-American Psychiatric Association, 2013; Craske and Stein, 2016; Roy-Byrne et al., 2006). To better understand the emotional alterations that characterise the behavioural profile of individuals suffering from panic disorder, animal models of fear have been used (Blanchard et al., 2001, 1988; Coimbra et al., 2017a, b; Paschoalin-Maurin et al., 2018). Studies have shown that both electrical and chemical stimulation of midbrain tectum structures, such as the periaqueductal grey matter (PAG) (Coimbra et al., 2006; Eichenberger et al., 2002; Graeff et al., 1986), the superior colliculus (SC) (Coimbra et al., 2006; da Silva et al., 2015; Eichenberger et al., 2002), and the inferior colliculus (IC) (Castilho et al., 1999; da Silva Soares et al., 2019), induce freezing and escape reactions that are considered panic attack-like behaviours (Blanchard et al., 1988; Calvo et al., 2019a; Coimbra et al., 2017a,b; Coimbra et al., 2006; dos Anjos-Garcia et al., 2017; Falconi-Sobrinho et al., 2017a,b).

Both the SC and the IC are part of the midbrain tectum, and although closely related, these structures have important morphological and functional differences (Mise, 1996). The corpora quadrigemina integrate sensorial and emotional functions (Castellan-Baldan et al., 2006; Coimbra et al., 2006; Osaki et al., 2003). The SC is a dorsal midbrain structure where visual, predatory, aversive and antinociceptive information are integrated to initiate innate fear-related responses (Coimbra et al., 2006; Coimbra and Brandão, 1993; Comoli et al., 2012, 2003). The IC acts primarily as an auditory nucleus involved in integrating and routing not only sensory perceptions but also defensive behaviours (Calvo et al., 2019b; Castellan-Baldan et al., 2006). The SC is an integrative sensorimotor structure that receives inputs from multiple sensory modalities and integrates them to control innate behaviours (Drager and David Hubel, 1975; Zingg et al., 2017). These characteristics suggest that the deep strata of the SC seem to play an important role in predatory hunting and innate defensive behaviours, such as escape (De Franceschi et al., 2016; Eichenberger et al., 2002; Furigo et al., 2010).

In addition, neurons situated in the deep layers of the SC (dlSC) have been suggested to be involved in adaptive defensive mechanisms related to pain control, such as fear-induced antinociception (Coimbra et al., 1992; Coimbra and Brandão, 1997; da Silva et al., 2015). Interestingly, the unconditioned fear-induced adaptive hypoalgesic response can be observed in animals exposed to aversive conditions, such as those related to confrontation against a natural predator (Coimbra et al., 2017a), and similar unconditioned fear-induced antinociception can be elicited after the activation of both the SC (Coimbra et al., 1992) and the IC (Castilho et al., 1999). Recently, da Silva Soares Jr. (2019) showed that intra-IC microinjections of NMDA in rats produce freezing and escape reactions, followed by unconditioned fear-induced antinociception that decreased after 5-hydroxytryptamine (5-HT)$_{2A}$ receptor blockade in the dorsal raphe nucleus (DRN).

Among the neurotransmitters that are involved in the processing of emotional responses, serotonin (or 5-HT) appears to play an important role in the regulation of defensive responses related to anxiety and fear (Deakin and Graeff, 1991; Graeff, 2003; Graeff et al., 1996). Serotonin is prominently found in the DRN, a mesencephalic structure that has been associated with the genesis and regulation of anxiety- and panic-like behaviours (Biagioni et al., 2013; Spiacci et al., 2012; Yamashita et al., 2017) and the mechanisms of pain control (Freitas et al., 2009; Kishi et al., 2006). DRN sends serotonergic projections to the dlSC (Graham, 1977; Janušonis et al., 1999; Villar et al., 1988; Waterhouse et al., 1986) and the IC, which seem to control the fear-related defensive responses organised by dorsal midbrain neurons (da Silva Soares et al., 2019).

Clinical and preclinical studies have demonstrated an important role for 5-HT$_{2A}$ receptors in the pathogenesis of panic disorder (De Oliveira Sergio et al., 2011; Inada et al., 2003; Pobbe and Zangrossi, 2005). For instance, the blockade of DRN 5-HT$_{2A}$ receptors with R-96544, a potent selective 5-HT$_{2A}$ receptor antagonist (Ogawa et al., 2002), caused a reduction in either panic-like behaviours or fear-induced antinociception elicited by the chemical stimulation of the IC (da Silva Soares et al., 2019). In addition, the increasing nociceptive threshold that accompanies these unconditioned fear-related reactions was also depressed by 5-HT$_{2A}$ receptor blockade in the DRN. This finding indicates that DRN 5-HT$_{2A}$ receptors are involved in the processing

of panic-like reactions and fear-induced antinociception organised by IC neurons. However, it is not clear whether DRN 5-HT_{2A} receptors modulate the defensive behaviour and fear-induced antinociception organised by the dlSC. Thus, the aim of the present study was to investigate the role of 5-HT_{2A} receptors in panic-like defensive reactions and fear-induced antinociception elicited by intra-dlSC microinjections of NMDA and to conduct the immunohistochemical characterisation of GABA and 5-HT_{2A} receptor distribution in DRN.

2. Experimental procedures

2.1. Animals

Male Wistar rats (*Rattus norvegicus* Rodentia, Muridae), 8–10 weeks old, weighing 250–300 g ($N = 72$, $n = 6$ per group) from the animal facility of Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP) were used. The rats were housed at 4–5 animals per cage with free access to water and food and maintained under a light/dark cycle of 12/12 h (lights on from 7 am to 7 pm) and at a constant room temperature of 24 °C ± 1 °C. The experiments were performed in accordance with the recommendations of the Commission of Ethics in Animal Experimentation of the FMRP-USP (process 017/2016), which is in agreement with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA) and the National Council for Animal Experimentation Control (CONCEA).

2.2. Stereotaxic surgery

The animals underwent deep anaesthesia with ketamine at 92 mg/kg (Ketamine Agener, *União Química Farmacêutica Nacional*, São Paulo, Brazil) and xylazine at 9.2 mg/kg (Calmium, *União Química Farmacêutica Nacional*, São Paulo, Brazil), and their heads were fixed in a stereotaxic frame (David Kopf, Tujunga, CA, USA). Stainless steel guide cannulae (outer diameter 0.6 mm, inner diameter 0.4 mm) were implanted ipsilaterally in the DRN and dlSC according to the following coordinates: DRN, anteroposterior (AP): −7.80 mm; mediolateral (ML): ± 0.2 mm; dorsoventral (DV): −5.4 mm and dlSC, AP: −6.96 mm; ML: −1.6 mm; DV: −3.6 mm, relative to bregma. The guide cannulae were fixed to the skull using acrylic resin and 1 stainless steel screw to protect the guide cannula lumen from obstruction. At the end of the surgery, each animal received an intramuscular injection of penicillin G benzathine (120,000 IU; 0.1 mL) and a subcutaneous injection of the non-steroidal analgesic and anti-inflammatory flunixin meglumine (2.5 mg/kg) (Schering-Plough, São Paulo, SP, Brazil).

2.3. Drugs

The N-methyl-D-aspartic acid receptor agonist (NMDA; Sigma/Aldrich, St. Louis, USA) at 6, 9 (da Silva Soares et al., 2019; Ullah et al., 2015) and 12 nmol (da Silva Soares et al., 2019) was dissolved in physiological saline (0.9% NaCl). The selective 5-HT_{2A} receptor antagonist (2R,4R)−5-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]−1-methyl-3-pyrrolidinol hydrochloride (R-96544; Tocris Bioscience, Avonmouth, Bristol, UK) at concentrations of 0.5, 5 and 10 nM (da Silva Soares et al., 2019) was dissolved in 10% dimethyl sulfoxide and saline (0.9% NaCl).

Aiming to avoid drug spreading toward the dorsolateral periaqueductal grey matter and to the inferior colliculus, NMDA was microinjected in the intermediate and deep strata of the superior colliculus at Bregma varying from −6.72 mm to −7.20 mm, avoiding reaching the superior colliculus at the level of the transition between cranial and caudal mesencephalon (from Bregma −7.56 mm to −7.80 mm). In addition, microinjections of R-96544 were perfomed in the dorsal and ventral subnuclei of the dorsal raphe nucleus (see Fig. 1C), avoiding the proximity of ventrolateral columns of the periaqueductal grey matter, the deep mesencephalon, and the aqueductus Sylvii. Finally, previous evidence demonstrated that the diffusion of chemical substances microinjected in the central nervous system into the tissue surrounding the site of injection is directly related to the volume administered (Myers, 1966; Routtenberg, 1972), and it has been suggested that 0.5 μL causes an average spread of 1.04 mm, consistent with Myers (1966). In this sense, to chemically activate the dorsal midbrain and to blockade DRN 5-HT_{2A} receptors of a rat, a droplet in the order of 0.5 μL is approximately the largest volume that can be used if an action of the drug is to be localised to that midbrain areas, according to Myers (1966). Thus, we assumed that volumes equivalent to 0.2 μL (volume used in the present study) reach a safe spherical area to justify the effect of the drug on the target structure (deep strata of the superior colliculus and dorsal raphe nucleus).

2.4. Psychopharmacological and morphological procedures

A timeline of each procedure was provided in Fig. 1.

2.4.1. Experiment 1: effect of the chemical stimulation of the dlSC with the NMDA receptor agonist on defensive behaviour and fear-induced antinociception

Five days after stereotaxic surgery, each animal was submitted to three measures of control tail-flick latencies to determine the baseline nociceptive threshold. Thereafter, independent groups of rats were randomly assigned to receive microinjections of 6, 9 and 12 nmol/0.2 μL NMDA or vehicle (0.9% NaCl/0.2 μL) into the dlSC. Microinjections were performed using a dental needle (0.3 mm OD) that was 1 mm longer than the guide cannulae, aiming at the dlSC. A polyethylene tube (PE-10) attached to a 5 μL syringe (Hamilton, Reno, Nevada, USA) connected to an infusion pump (Stoelting, Kiel, Wisconsin, USA) was used to inject the drug (a volume of 0.2 μL over 15 s). To prevent reflux, the dental needle was left in place for 30 s after the end of each injection. After the intra-dlSC microinjection of NMDA, the behavioural responses were quantitatively analysed for 10 min. To perform the behavioural test, a circular arena (50 cm x 60 cm) with transparent acrylic was used, situated in an illuminated experimental environment (617 lx at the circular arena floor level). Following the behavioural tests, the nociceptive thresholds were measured at 10-min intervals for 70 min.

2.4.2. Experiment 2: effects of the 5-HT_{2A} receptor antagonist R-96544 on the defensive behaviour and fear-induced antinociception elicited by the chemical stimulation of the dlSC

After a postoperative period of five days, the baseline nociceptive threshold of each animal was determined using the tail-flick test. On the day of the experiment, each animal was pretreated with microinjections of 0.5, 5 and 10 nM R-96544 or vehicle into the DRN. Ten minutes later, NMDA at 12 nmol/0.2 μL was microinjected into the dlSC. The microinjection procedure was similar to that used in the previous experiment. After the administration of NMDA into the dlSC, the defensive responses displayed by the rats in the circular arena were quantitatively analysed for 10 min, and immediately after the behavioural tests, the tail-flick withdrawal latencies were measured at 10-min intervals for 70 min.

2.4.3. Behavioural analysis

Behavioural responses were recorded for 10 min using a video camera (Handycam, Sony Corporation, Osaki, Shinagawa-ku, Tokyo,

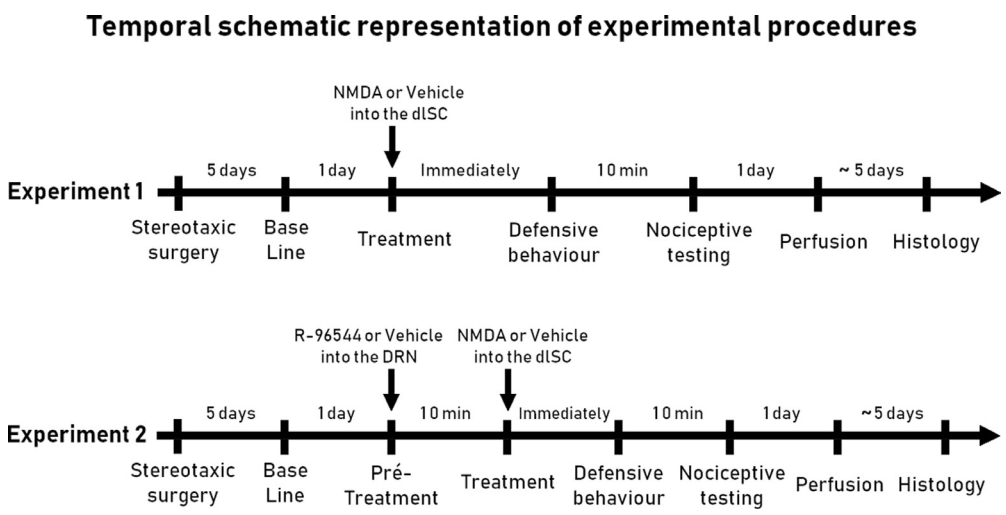

Fig. 1 Timeline of each procedure performed in the present work.

Japan). The behavioural tests were performed in a circular arena with transparent acrylic walls. The floor of the circular arena was divided into twelve equal sections used to count crossings. Behavioural defensive reactions were quantified by measuring the number and duration of freezing behaviour, which is characterised by defensive immobility for at least 6 s, followed by a visible autonomic reaction, such as defecation, exophthalmia and/or micturition (Falconi-Sobrinho and Coimbra, 2018). An escape response was defined as running interspersed with jumping and quantified by measuring the number and duration of escape events (Biagioni et al., 2016, 2013). In addition, the number of crossings, characterised as 4 paws within a given division of the floor of the circular arena, was used as a quantitative measure of exploratory behaviour (da Silva et al., 2015). The researcher who performed the experiments was the same who analysed the behavioural data.

2.4.4. Nociceptive testing

The nociception thresholds of the rats were compared using the tail-flick test. Each animal was placed in a restraining apparatus (Insight, Ribeirão Preto, Brazil) with acrylic walls, and its tail was placed on a heating coil (tail-flick Analgesia Instrument; FMRP-USP Precision Workshop, Ribeirão Preto, Brazil). The amount of heat applied to the tail was increased until the animal removed its tail from the apparatus. The coil (Ni/Cr alloy; 26.04 cm in length × 0.02 cm in diameter) was initially maintained at room temperature (approximately 20 °C), and then current was applied to increase the temperature of the coil at a rate of 9 °C/s (Falconi-Sobrinho et al., 2017a,b). If necessary, small adjustments were made to the intensity of the current at the beginning of the experiment to obtain three consecutive tail-flick latencies (TFL) between 2.5 and 3.5 s. If the animal did not remove its tail from the heater within 6 s, then the apparatus was turned off to prevent damage to the skin. Three baseline measurements of control TFL were taken at 5-min intervals. TFL was also measured every 10 min for 70 min (t0, t10, t20, t30, t40, t50, t60, and t70) immediately after the end of the defensive behaviour assay.

2.4.5. Experiment 3: morphological localisation of 5-HT$_{2A}$ receptors and GABAergic neurons in the DRN

The immunofluorescence technique was performed as described elsewhere (Almada et al., 2015). Briefly, slices of mice encephalon were incubated in 0.1 M sodium phosphate buffer (LabSynth, Diadema, São Paulo, Brazil; pH 7.2) overnight. The next day, antigen retrieval with 10 M sodium citrate (pH 6.0) was performed for 30 min in a water bath at 40 °C. The sections were washed three times with 0.1 M sodium phosphate buffer for 5 min each and 0.1 M glycine (Sigma-Aldrich) for 30 min. The sections were incubated with image-IT (Life Technologies, Carlsbad, CA, USA) for 1 h and simultaneously incubated with the following primary antibodies: rabbit anti-5-HT$_{2A}$ receptor IgG (1:200 dilution, Abcam Plc, Cambridge, UK), and mouse anti-GABA IgG (1:200 dilution, Sigma-Aldrich). Neural tissue sections were washed three times for 5 min each, simultaneously incubated with secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG 1:500 and Alexa Fluor 568 goat anti-mouse IgG, Invitrogen, Carlsbad, CA, USA) for 120 min in the dark, and washed again three more times. Finally, the slides were coverslipped with Prolong with DAPI (Life Technologies), and histological sections were analysed by motorised microscopy (AxioImager Z1 with APOTOME, Zeiss, Oberkochen, Germany).

2.5. Histology

Upon completion of the experiments, each animal was anaesthetised with ketamine at 92 mg/kg (Ketamina®) and xylazine at 9.2 mg/kg (Dopaser®) and perfused through the left cardiac ventricle using an infusion pump (Master Flex® L/S TM, Vernon Hills, IL, USA). The animal was then perfused with paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 15 min. The encephalon was quickly removed and maintained in 4% paraformaldehyde for at least 4 h and was then immersed in a 10% and 20% sucrose solution for 12 h in each solution. Tissue pieces were immersed in 2-methylbutane (Sigma-Aldrich), frozen on dry ice (30 min), embedded in Tissue-Tek, and cut on a cryostat (CM 1950, Leica, Mannheim, Germany). The slices were then mounted on glass slides coated with chrome alum gelatine to prevent detachment and stained in a robotic autostainer (CV 5030 Leica Autostainer) with haematoxylin-eosin. The sections were viewed under a motorised photomicroscope (AxioImager Z1, Zeiss), and the positions of the tips of the guide cannulae were localised according to stereotaxic atlas (Paxinos and Watson, 2007). The data from rats with guide cannulae tips located outside of their target regions (SC and/or DRN) were not included in the statistical analysis.

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., California, USA). The data from independent groups were subjected to the Shapiro-Wilk test of normality

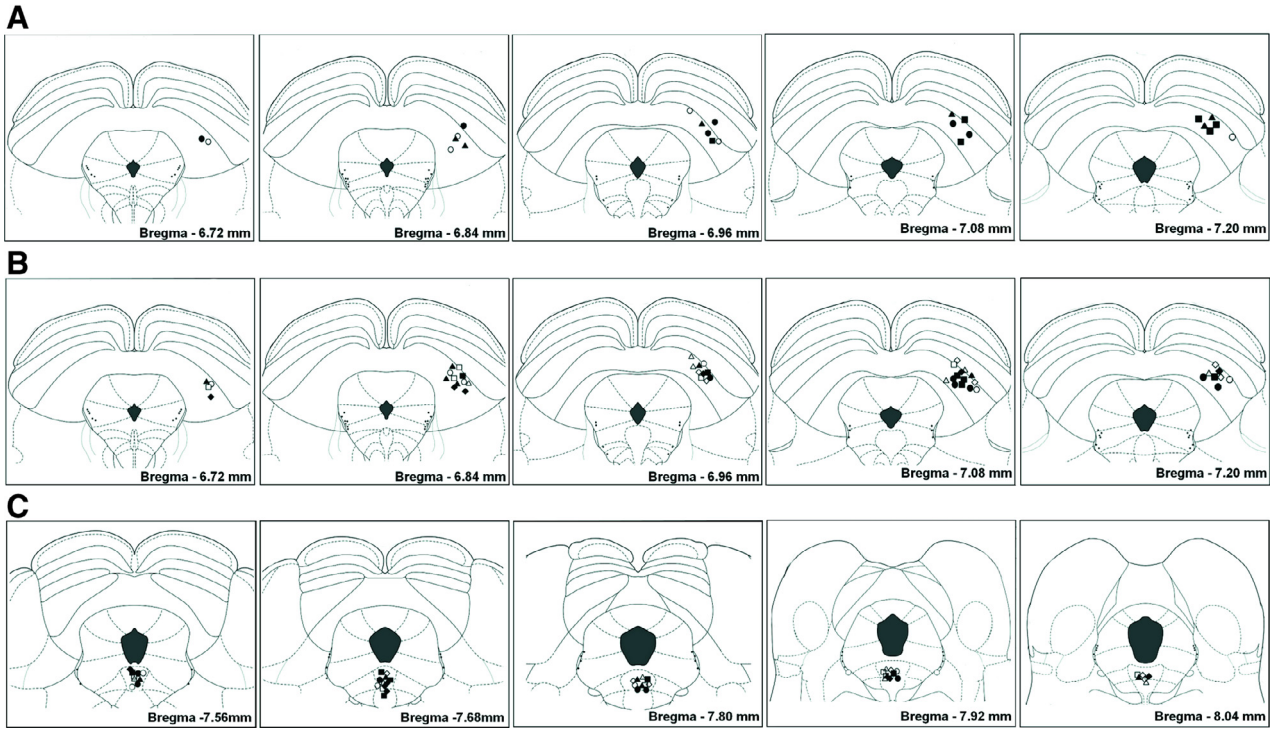

Fig. 2 A: Diagrammatic representation of transverse sections of the rat midbrain showing histologically confirmed sites of microinjections of the following drugs: (A) NMDA at (■) 6 nmol/0.2 μL, (▲) 9 nmol/0.2 μL, (●) 12 nmol/0.2 μL or vehicle (○) into the deep layers of the superior colliculus (dlSC). (B) vehicle into the dorsal raphe nucleus (DRN) + NMDA at (●) 12 nmol/0.2 μL in the dlSC, R-96544 at (♦) 0.5 nM/0.2 μL, (■) 5 nM/0.2 μL, or (▲) 10 nM/0.2 μL in the DRN + NMDA at 12 nmol/0.2 μL in the dlSC, (○) vehicle in the DRN + vehicle in the dlSC, R-96544 at (◊) 0.5 nM/0.2 μL, (□) 10 nM/0.2 μL, and (△) 10 nmol/0.2 μL in the DRN + vehicle in the dlSC. (C) Histologically confirmed sites of the microinjection of drugs in the DRN.

and analysed by a parametric test since the data fit within Gaussian distributions and the variances between groups were homogeneous for more than 50% of the data. One-way analysis of variance (one-way ANOVA), followed by Tukey's *post hoc* tests was used to analyse the behavioural studies in experiment 1. Two-way analysis of variance (two-way ANOVA), followed by Tukey's *post hoc* tests was used to analyse the behavioural studies in experiment 2. Two-way repeated measures analysis of variance (two-way RM-ANOVA), followed by Tukey's multiple comparison test was used to analyse the tail-flick latencies in both experiments. In all cases, the data are expressed as the means ± S.E.M. In all cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Experiment 1: microinjections of NMDA into the dlSC elicited defensive behaviour and fear-induced antinociception

Histologically confirmed sites of NMDA (6, 9 and 12 nmol) or vehicle microinjections into the dlSC are shown as schematic drawings of transverse sections in Fig. 2A.

3.1.1. Defensive behaviour

According to one-way ANOVA, followed by Tukey's *post hoc* test, there was a significant effect of treatment on the frequency [$F_{(3,20)} = 15.98$; $p < 0.001$] and duration [$F_{(3,20)} = 4.808$; $p < 0.05$] of freezing. Intra-dlSC treatment with NMDA at 6, 9 and 12 nmol elicited a dose-dependent response in the frequency ($p < 0.01$, $p < 0.001$ and $p < 0.01$, respectively) and duration (Tukey's *post hoc* test; $p < 0.05$) of freezing behaviour (Fig. 3A and B).

Regarding escape behaviour, which was characterised by running interspersed with jumps reactions, there was a significant effect of treatment on the frequency [$F_{(3,20)} = 3.895$; $p < 0.05$] but not the duration [$F_{(1568,\ 7841)} = 3.778$; $p > 0.05$], according to one-way ANOVA. Only the highest dose (12 nmol) of NMDA microinjected into the dlSC showed a significantly different effect from that in the vehicle-control group on the frequency ($p < 0.05$) of escape behaviour, according to Tukey's *post hoc* test (Fig. 3C and D). In addition, according to one-way ANOVA, there was no significant effect of treatment on the frequency [$F_{(3,20)} = 2.203$; $p > 0.05$] of crossings (Tukey's *post hoc* test; $p < 0.05$) (Fig. 3E).

3.1.2. Fear-induced antinociception

The activation of dlSC neurons with NMDA at all doses evoked unconditioned fear-induced antinociception in rodents. According to two-way RM-ANOVA, there were statistically significant effects of treatment [$F_{(3,20)} = 3.767$; $p < 0.05$], time [$F_{(8160)} = 42.42$; $p < 0.001$], and a treatment versus time interaction [$F_{(24,160)} = 3.938$; $p < 0.001$]. The group of rats that received intra-dlSC microinjections of NMDA at the highest dose (12 nmol) showed antinociception at zero to 20 min after the demonstration of panic-like

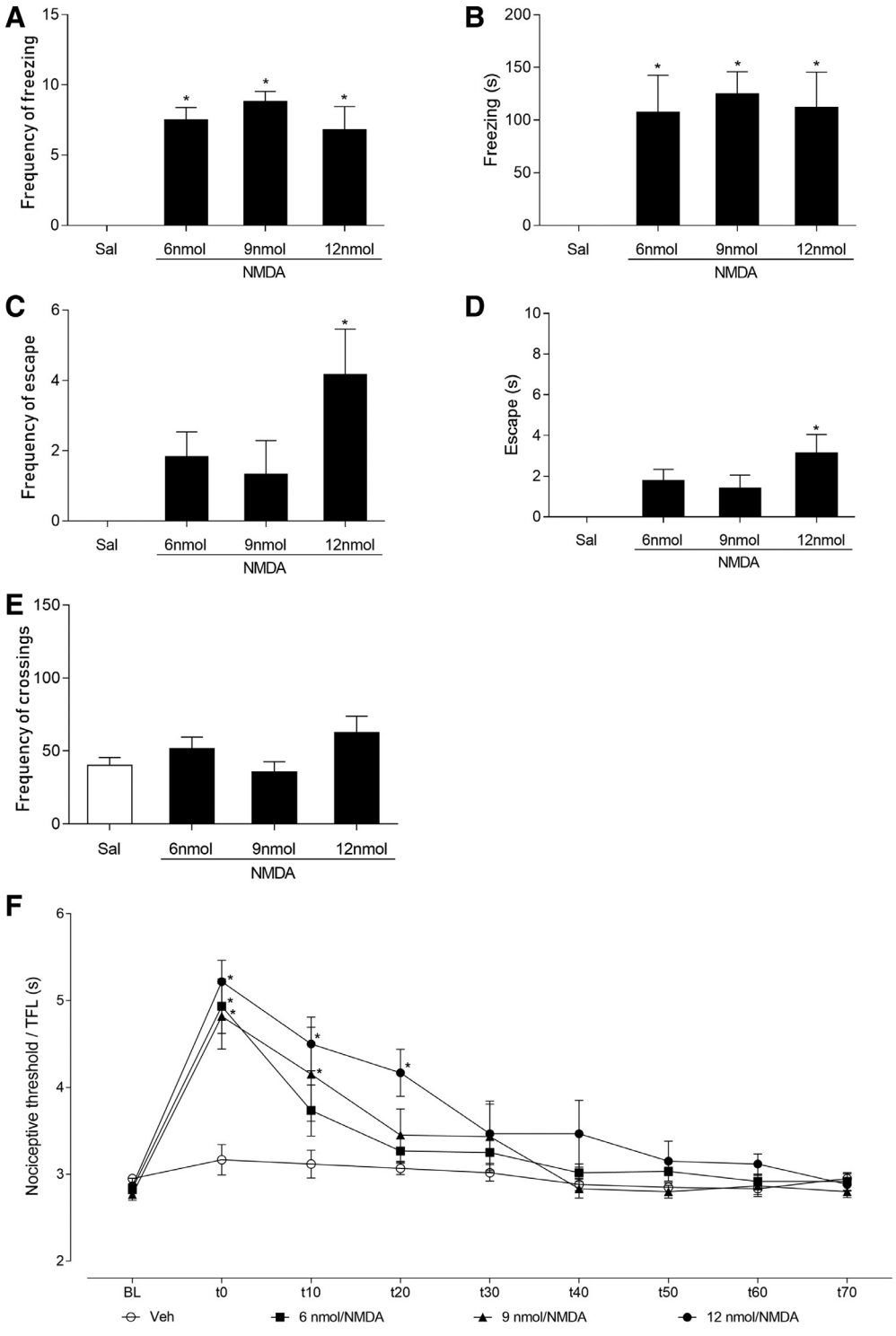

Fig. 3 Effect of central microinjections of NMDA (6, 9 and 12 nmol/0.2 μL) or vehicle (NaCl 0.9%; 0.2 μL) into the superior colliculus (SC) on the frequency (number of behavioural events; A, C and E) and duration (B and D) of freezing (A and B), escape (C and D) and crossings (E); $n = 6$ per group. The columns represent the means, and the bars represent the standard error of the mean; $^{*}p < 0.05$ compared with the vehicle (veh)-treated control group (according to one-way ANOVA followed by Tukey's *post hoc* test). (F) Effect of the central microinjection of NMDA (6, 9 and 12 nmol/0.2 μL) or vehicle (NaCl 0.9%; 0.2 μL) into the SC on tail-flick latencies. Antinociception caused by defensive behaviour was recorded using the tail-flick test; $n = 6$ per group. Data are presented as the means ± standard error of the mean; $^{*}p < 0.05$ compared with the vehicle (veh)-treated control group, according to two-way RM-ANOVA, followed by Tukey's multiple comparison tests.

defensive behaviours, while the rodents receiving NMDA at the intermediate dose (9 nmol) and the lowest dose (6 nmol) showed an increase in tail withdrawal latency at zero to 10 min and immediately after escape (time zero), respectively, when compared to the vehicle-control group (Tukey's *post hoc* tests; $p < 0.05$ in all cases) (Fig. 3F).

3.2. Experiment 2: blockade of the 5-HT$_{2A}$ receptor in the DRN promoted a decrease in the defensive behaviour and fear-induced antinociception organised by dlSC neurons

Histologically confirmed sites of R-96544 (0.5, 5 and 10 nM) or vehicle microinjections into the DRN and NMDA (12 nmol) or vehicle microinjections into intra-dlSC are shown as schematic drawings of midbrain transverse sections in Fig. 2B and C.

3.2.1. Defensive behaviour

According to two-way ANOVA, there was a statistically significant effect of the DRN [$F_{(3.40)} = 35.71$; $p < 0.001$] and dlSC [$F_{(1.40)} = 78.35$; $p < 0.001$] treatments and of the interaction between these factors [$F_{(3.40)} = 35.71$; $p < 0.001$] regarding the incidence (number) of freezing. Similar to the first set of experiments, according to two-way ANOVA, the dlSC treatment with NMDA at 12 nmol evoked freezing, which was statistically expressed by the increased frequency ($p < 0.001$) and duration ($p < 0.001$) of freezing behaviour compared with the control group. Pretreatment of the DRN with R-96544 at different concentrations (0.5, 5 and 10 nM) decreased both the number (Tukey's *post hoc* test; $p < 0.001$ in all cases) and duration (Tukey's *post hoc* test; $p < 0.001$ in all cases) of freezing behaviours evoked by NMDA microinjections into the dlSC. Intra-DRN treatment with R-96544 at different concentrations, followed by the administration of physiological saline into the dlSC had no effect on the frequency and duration (Tukey's *post hoc* test; $p > 0.05$ in both cases) of freezing compared with the control group, as shown in Fig. 4A and B.

Regarding escape behaviour, according to two-way ANOVA, there was a statistically significant effect of the DRN [$F_{(3.40)} = 3.946$; $p < 0.05$] and dlSC [$F_{(1.40)} = 22.04$; $p < 0.001$] treatments and of the interaction between these factors [$F_{(3.40)} = 3.946$; $p < 0.05$]. Compared with the vehicle + vehicle-treated control group, intra-dlSC microinjections of NMDA elicited a greater number and longer duration of escape (Tukey's *post hoc* test; $p < 0.001$ in both cases). Pretreatment of the DRN with R-96544 at higher concentrations (5 and 10 nM) significantly decreased the frequency (Tukey's *post hoc* test; $p < 0.01$ in both cases) and duration (Tukey's *post hoc* test; $p < 0.05$ in both cases) of escape behaviour, while the lower concentration of R-96544 was able to decrease only the duration of escape (Tukey's *post hoc* test; $p < 0.05$), as shown in Fig. 4C and D.

Compared with the control group, the pretreatment of the DRN with R-96544 at 0.5, 5 and 10 nM *per se* showed no intrinsic effect on either freezing or escape behaviours (Tukey's *post hoc* test; $p > 0.05$ in all cases) (Fig. 4A and D).

Regarding the motor behaviour of crossing displayed by rodents during the exploratory behaviour, according to two-way ANOVA, there was a statistically significant effect of dlSC [$F_{(1.40)} = 6.791$; $p < 0.05$] but not DRN [$F_{(3.40)} = 1.482$; $p > 0.05$] treatments, and there was no effect of the interaction between these factors [$F_{(3.40)} = 2.814$; $p > 0.05$]. The group of rats that received intra-dlSC microinjections of NMDA, followed by the administration of vehicle in the DRN did not exhibited more crossing (Tukey's *post hoc* test; $p > 0.05$) than did the control group during the exploratory behaviour. Compared with vehicle + NMDA treatment, pretreatment of the DRN with R-96544 at different concentrations (0.5, 5 and 10 nM) was not able to decrease the frequency of exploratory behaviour-related crossings (Tukey's *post hoc* test; $p > 0.05$ in all cases) (Fig. 4E).

3.2.2. Fear-induced antinociception

Similar to the first experiment, the panic-like defensive behaviour elicited by treatment of the dlSC with NMDA was followed by an antinociceptive response. In addition, pretreatment of the DRN with R-96544 significantly decreased the unconditioned fear-related adaptive hypoalgesic response. According to two-way RM-ANOVA, there were statistically significant effects of treatment [$F_{(7,40)} = 28.3$, $p < 0.001$], time [$F_{(8320)} = 35.1$, $p < 0.001$] and interaction between treatment versus time [$F_{(56,320)} = 14.9$, $p < 0.001$]. Compared with the control group, the activation of neurons localised in the dlSC by local microinjections of NMDA preceded by vehicle administered into the DRN increased the tail-flick latencies during the first 40 min after the panic-like defensive behaviour (Tukey's multiple comparison test; $p < 0.001$). Compared with the vehicle + NMDA-treated group and the R-96544 at 0.5 nM + NMDA-treated group (Tukey's post hoc test; $p < 0.001$ in both cases), intra-DRN pretreatment with R-96544 at 5 and 10 nM decreased unconditioned fear-induced antinociception, as shown in Fig. 4F.

The rodents receiving intra-DRN microinjections of R-96544, followed by vehicle administration in the dlSC, displayed no evidence of an intrinsic effect of the intra-mesencephalic treatment on tail-flick latencies (Tukey's post hoc test; $p > 0.05$), as shown in Fig. 5F.

3.3. Experiment 3: 5-HT$_{2A}$ receptors are located on presynaptic GABAergic neurons in the DRN

We found 5-HT$_{2A}$ receptor labelling localised on the perikaryon of GABAergic cells in both the DRD (dorsal sub-region) and WRD (lateral wing sub-region) divisions of the DRN, suggesting that 5-HT$_{2A}$ receptors are postsynaptically located on GABAergic neurons in the DRN, potentially acting in the modulation of GABAergic neural firing (Fig. 5). Interestingly, GABA labelling was identified in both cellular bodies and in neuronal fibres as puncta surrounding GABAergic perikarya in both the DRD (Fig. 5B) and WRD divisions (Fig. 5F), with a predominant distribution of fibres situated in the dorsal raphe lateral wing region. The specificity of the antibodies used in the present work has been demonstrated elsewhere (Wei et al., 2010; Zhang et al., 2016).

4. Discussion

The present findings demonstrate that the chemical stimulation of dlSC neurons with NMDA elicited freezing and

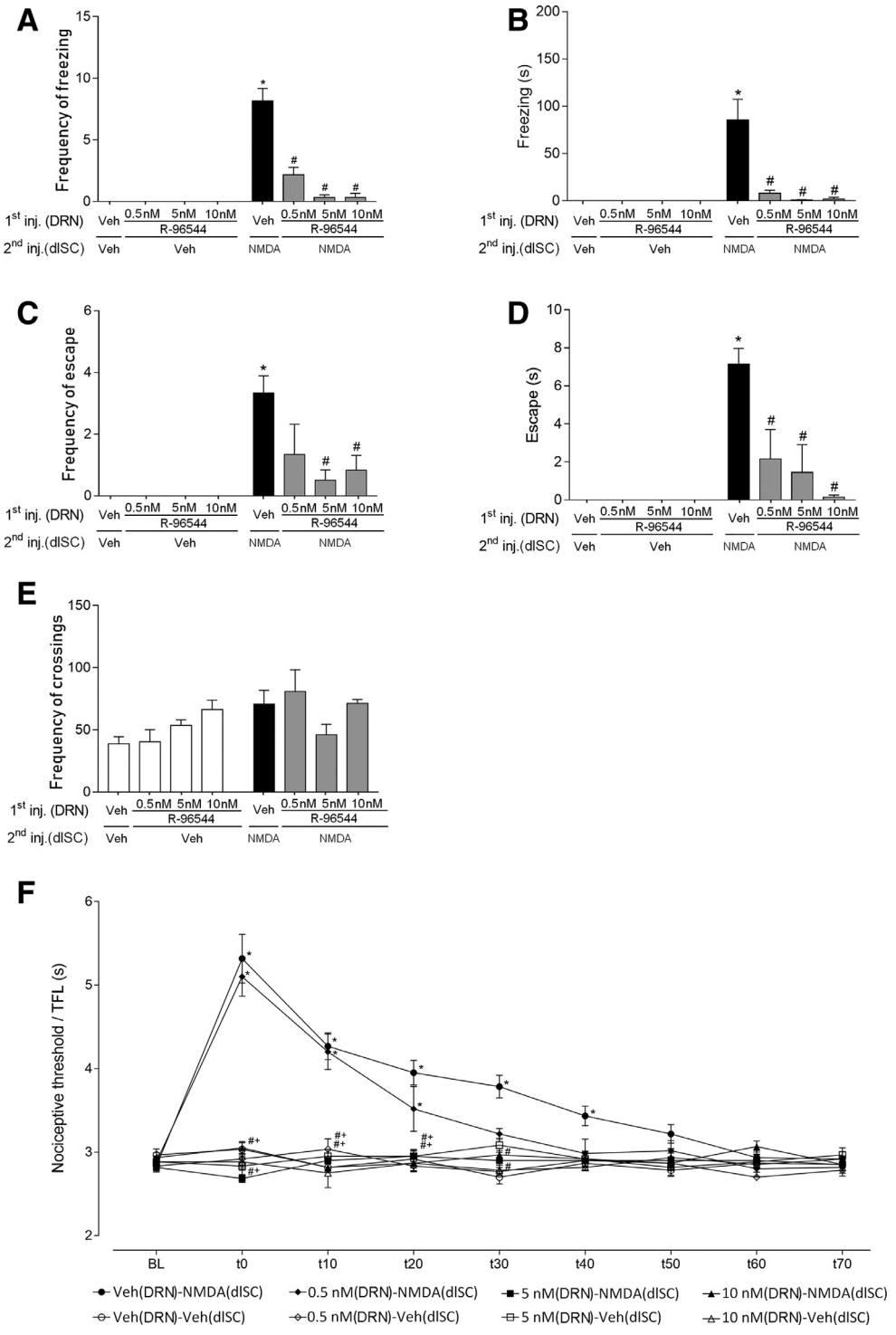

Fig. 4 Effect of the central microinjection of R-96544 (0.5, 5 and 10 nM/0.2 μL) or vehicle (10% DMSO) into the dorsal raphe nucleus (DRN) on the frequency (number of behavioural events; A, C and E) and duration (B and D) of freezing (A and B), escape (C and D) and crossings (E) evoked by a microinjection of NMDA at 12 nmol/0.2 μL or vehicle (NaCl 0.9%;0.2 μL) into the superior colliculus (SC); $n = 6$ per group. The columns represent the means, and the bars represent the standard error of the mean; $^{*}p < 0.05$ compared with the vehicle (veh)-veh-treated control group; $\#p < 0.05$ compared with the veh-NMDA-treated group (according to two-way ANOVA followed by Tukey's *post hoc* test). (F) Effect of the central microinjections of R-96544 (0.5, 5 and 10 nM/0.2 μL) or vehicle (10% DMSO) into the DRN followed by a microinjection of NMDA at 12 nmol/0.2 μL or vehicle (NaCl 0.9%; 0.2 μL) into the SC on unconditioned fear-induced antinociception. Antinociception caused by defensive behaviour was recorded using the tail-flick test; $n = 6$ per group. The data are presented as the means ± standard error of the mean; $^{*}p < 0.05$ compared with the vehicle (veh)/veh-treated control group; $\#p < 0.05$ compared with the veh/NMDA-treated group; $+p < 0.05$ compared with the R-96544 at 0.5 nM/0.2 μL (0.5 nM)/NMDA-treated group, according to two-way RM-ANOVA, followed by Tukey's multiple comparison test.

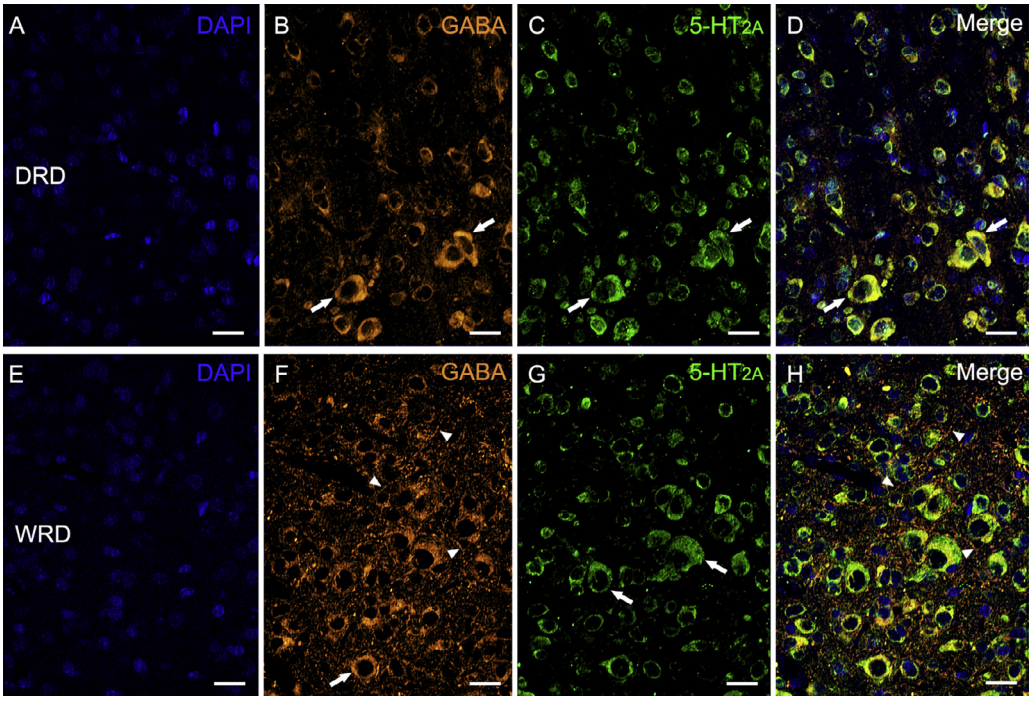

Fig. 5 Photomicrographs of transverse sections of the dorsal (DRD) and lateral wings (WRD) of the dorsal raphe nucleus (DRN), showing immunohistochemically labelled gamma-aminobutyric acid (GABA)- and 5-hydroxytryptamine (5-HT)$_{2A}$ receptor-containing fibres and perikarya.

escape defensive reactions, followed by an increase in the nociceptive threshold. We demonstrated that the blockade of 5-HT$_{2A}$ receptors in the DRN significantly reduced both defensive reactions and unconditioned fear-induced antinociception evoked by microinjections of NMDA into the dlSC. Our results confirm and provide new evidence regarding the serotonergic modulation of instinctive fear-related defensive behavioural and antinociceptive responses organised by midbrain tectum neurons.

Several laboratory animal-based studies have shown that intense fear is capable of inducing an adaptive antinociceptive response (Coimbra et al., 2017a, b, 2006; Coimbra and Brandão, 1993; Falconi-Sobrinho et al., 2017; Fanselow, 1986). Here, we observed that increasing doses of NMDA microinjected into the dlSC promoted an intense defensive behavioural reaction that was accompanied by an expressive antinociceptive response. Rats treated with NMDA at the highest dose (12 nmol) in the dlSC exhibited freezing and vigorous escape behaviour that was followed by an increase in the nociceptive threshold up to 20 min after the end of the panic attack-like behaviours. Interestingly, our findings demonstrated that the defensive behavioural patterns exhibited by rodents that received intra-SC microinjections of NMDA at higher doses were similar to the fearful behavioural reactions triggered by animals that received the same doses of NMDA in the IC (da Silva Soares et al., 2019). However, the animals that received NMDA in the IC showed a longer duration of antinociception, recorded at 40 min after the end of the defensive escape behaviour.

Furthermore, the increased activity of SC excitatory neurons by local chemical stimulation with NMDA promotes proaversive effects similar to those exhibited by rodents subjected to electrical stimulation in mesencephalic structures (Castilho and Brandão, 2001; Coimbra et al., 2006, 1992; Nashold et al., 1969). For instance, Coimbra and Brandão (1997) demonstrated that electrical stimulation in both the dlSC and PAG provoked vigorous defensive reactions expressed by freezing, running, and increased nociceptive thresholds. Both panic-like behaviour and unconditioned fear-induced antinociception consist of evolutionarily preserved defensive responses triggered to promote the survival of animals when they are exposed to dangerous situations (Blanchard et al., 2013; Coimbra et al., 2017a, b; Lojowska et al., 2015). Almada et al. (2015) showed that rodents confronted with snakes display panic-like defensive reactions. Interestingly, a recent study by Coimbra et al. (2017a) demonstrated that rats confronted with venomous snakes showed an increase in the nociceptive threshold after they displayed defensive behaviours. At least some of the defensive hypoalgesia displayed by rats in threatening situations such as those (Coimbra et al., 2017a), seem to be at least partially organised by dorsal midbrain structures (Coimbra et al., 2006, 1992; Coimbra and Brandão, 1997) and the pain endogenous modulatory system neural network, such as the gigantocellularis/paragigantocellularis pars alpha reticular nuclei, the nucleus raphe magnus (de Oliveira et al., 2017) and the DRN (da Silva Soares et al., 2019), which is associated with the dlSC and dlPAG (Coimbra et al., 2006).

Interestingly, intra-DRN microinjections of the 5-HT$_{2A}$ receptor selective antagonist R-96544 also attenuated the panic-like defensive behaviour organised by the dlSC. The 5-HT$_{2A}$ receptor is a Gq-coupled heteroreceptor that

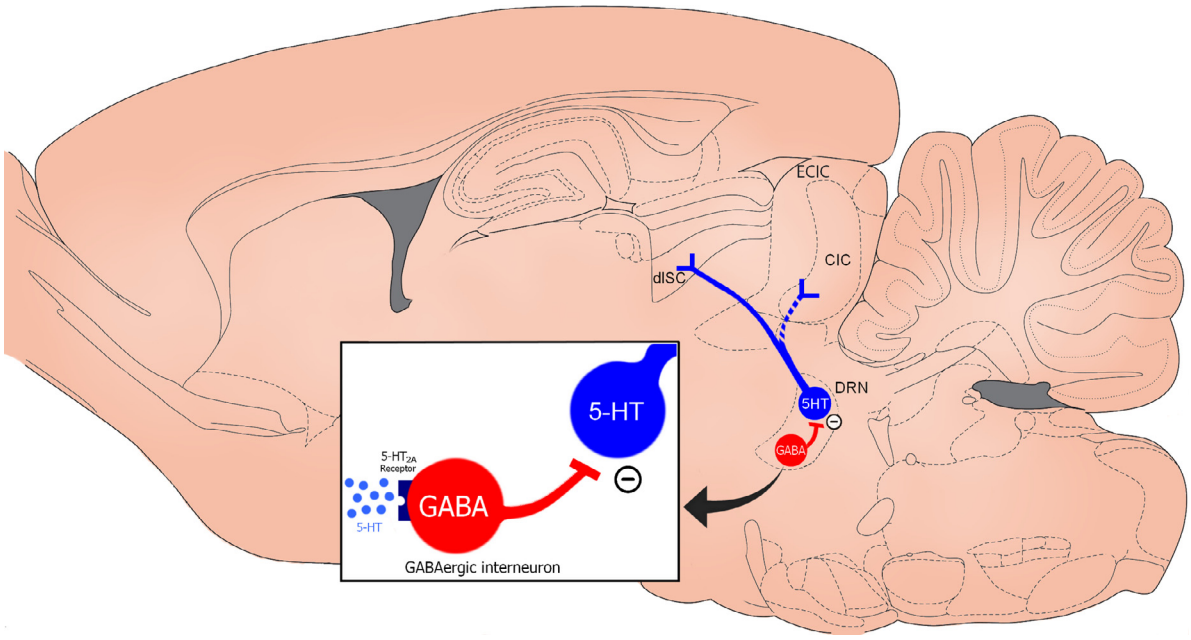

Fig. 6 Schematic diagram suggesting the modulatory role of GABAergic interneurons activity via 5-hydroxytryptamine (5-HT)$_{2A}$ receptors, showing an interaction between the GABAergic inhibitory neurons (red) and dorsal raphe nucleus (DRN) efferent connexions (blue) within the deep layers of the superior colliculus (dlSC) and the central nucleus (CIC) and external cortex (ECIC) of the inferior colliculus. The schematic parasagittal section of the rat brain is a modified drawing from Paxinos and Watson's rat brain in stereotaxic coordinates atlas (2007).

regulates the synthesis and/or release of mediators other than its own ligands (Barnes and Sharp, 1999). The morphological approaches performed in the present study demonstrated an overlapping of GABAergic neurons and 5-HT$_{2A}$ receptors in both the DRD and WRD, suggesting that DRN 5-HT$_{2A}$ receptors are postsynaptically localised on GABAergic neuronal perikarya, although GABA-labelling was found on both neurites and neuronal bodies in the DRN. In addition, there is evidence that the DRN sends 5-HT projections to the other regions of the midbrain tectum, such as the SC (Edwards et al., 1990; Vertes, 1991; Villar et al., 1988). Considering that the blockade of 5-HT$_{2A}$ receptors by local injections of R-96544 decreases the activity of GABAergic interneurons that would inhibit serotonergic projections to the midbrain tectum, we can argue that the increased activity of DRN-SC serotoninergic ascending pathways, possibly those from the DRN lateral wings (Villar et al., 1988), by local GABAergic disinhibition, attenuated the freezing and escape behaviours organised by the SC (Fig. 6).

Antiaversive effects in other regions of the mesencephalon, such as the dPAG, promoted by local 5-HT release due to the increased activity of 5-HT pathways from the DRN, has also been reported elsewhere (Pobbe and Zangrossi, 2005). These authors showed that the blockade of the 5-HT$_{1A}$ receptor in the DRN by local injections of the 5-HT$_{1A}$ receptor antagonist WAY-100635 impairs the escape reactions triggered by rats in the elevated T-maze test. In addition, the panicolytic-like effect promoted by intra-DRN treatment with WAY-100635 was abolished by pretreatment of the dPAG with ketanserin, a 5-HT$_{2A/2C}$ receptor preferential antagonist. Notably, 5-HT$_{1A}$ is an autoreceptor responsible for the inhibitory effects of adenylate cyclase intracellular neurochemical mechanisms. This increase in serotonergic activity has been suggested to promote panicolytic-like effects (Graeff, 2003).

Taken together, these data reinforce the hypothesis that the increased activity of DRN-collicular 5-HT ascending pathways attenuates panic-like reactions in laboratory rats. Moreover, the activity of serotonergic neurons from the DRN seems to be recruited by either the activation of 5-HT$_{2A}$ receptors or the inhibition of 5-HT$_{1A}$ receptors in the DRN. We also demonstrated that the antinociceptive response following defensive reactions provoked by intra-dlSC injections of NMDA was also impaired by 5-HT$_{2A}$ receptor blockade in the DRN. Considering that the increase in the nociceptive threshold is related to the stressor context (i.e., unconditioned fear), we can argue that if the treatment of the DRN with R-96544 diminishes dlSC-mediated panic-like defensive behaviours, then it is expected that these animals do not exhibit a robust antinociceptive response. However, recent evidence for the dissociation between mechanisms involving defensive behaviour and fear-induced antinociception has previously been reported (da Silva et al., 2015). These authors suggested that the activation of the CB$_1$ receptor by injections of cannabidiol (CBD) into the substantia nigra pars reticulata attenuates panic-like behaviours but does not alter the unconditioned fear-induced antinociception provoked by the GABAergic disinhibition of dlSC neurons. In addition, studies have suggested that the panicolytic-like effects promoted by CBD microinjected into the dorsal mesencephalon, such as the PAG, depend on the activation of local 5-HT$_{1A}$ (De Paula Soares et al., 2010), a receptor that when blocked in the medial hypothalamus attenuates a fear-induced antinociceptive response

(Biagioni et al., 2016b). Interestingly, Coimbra and Brandão (1997) demonstrated that the blockade of 5-$HT_{2A/2C}$ receptors by intra-mesencephalic microinjections of ketanserin impaired the unconditioned fear-induced antinociception provoked by electrical stimulation of the midbrain tectum without causing any significant changes in freezing and escape thresholds.

Clinical studies suggest the $5HT_{2A}$ receptor as an important target for the better understanding of the neuropharmacological bases of panic syndrome and its treatment. Inada and co-workers (2003) investigated the association of panic syndrome with HT_{1A}, HT_{2A} and HT_{2C} DNA markers in biologically unrelated patients with panic disorder. Polymorphisms of the HT_{1A}, HT_{2A} and HT_{2C} genes were determined using polymerase chain reaction and analysis of restriction fragment-length polymorphisms. These authors showed a positive association between panic syndrome and the HT_{2A} gene, suggesting that the HT_{2A} receptor plays a relevant role in the pathogenesis of panic syndrome. The present work has a considerable translational value when reinforces the idea that the 5-HT_{2A} receptor consists in a putative pharmacological target for the treatment of panic disorder. Further investigations can deeper investigate the role played by the connexions between the DRN and other structures of the brain aversion system elucidating the relevance of DRN 5-HT_{2A} receptors in the modulation of panic attacks in humans.

In conclusion, our findings demonstrate that the blockade of DRN 5-HT_{2A} receptors decreased both panic attack-like defensive behaviour and unconditioned fear-induced antinociception organised by the dlSC, possibly by activating ascending pathways from the DRN to the dlSC.

CRediT authorship contribution statement

Raimundo da Silva Soares Jr.: Investigation, Formal analysis, Writing - original draft, Validation. **Luiz Luciano Falconi-Sobrinho:** Investigation, Formal analysis, Writing - original draft, Validation. **Rafael Carvalho Almada:** Writing - review & editing, Validation. **Norberto Cysne Coimbra:** Conceptualization, Investigation, Funding acquisition, Project administration, Supervision, Writing - original draft, Validation.

Acknowledgements

R da Silva-Soares Jr. was supported by CAPES (M.*Sc*. fellowship process PROEX0053040). R.C. Almada was a postdoctoral researcher supported by FAPESP (grant 2012/03798-0) and CAPES (grant PNPD-20131680-33002029012P3). N.C. Coimbra was awarded a research fellowship (level 1A) from the CNPq (grants 301905/2010-0 and 301341/2015-0). The authors are grateful to D.H. Elias-Filho for providing expert technical assistance. D.H. Elias-Filho received a technician scholarship from FAPESP (TT-2, process 02/01497-1) and was the recipient of scholarships sponsored by the CNPq (processes 501858/2005-9, 500896/2008-9, 505461/2010-2, and 372838/2018-9) and the FAEPA (grants 345/2009 and 185/2010).

Role of funding source

This study was supported by the *Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico* (CNPq) (grants 483763/2010-1, 474853/2013-6, and 427397/2018-9), *Fundação de Apoio ao Ensino, Pesquisa e Assistência do HC-FMRP-USP* (FAEPA) (grants 1291/1997, 355/2000, 68/2001, and 15/2003) and the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP) (grants 1995/3604-4, 1995/8418-4, 2007/01174-1, and 2012/03798-0).

Declaration of Competing of interest

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

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