Physiology & Behavior 179 (2017) 504–509

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/physbeh

Cannabinoid system of dorsomedial telencephalon modulates behavioral responses to noxious stimulation in the fish *Leporinus macrocephalus*

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ARTICLE INFO

Keywords:
Amygdala
Cannabidiol
CB1
Nociception

ABSTRACT

Fish dorsomedial telencephalon has been considered a pallial region homologous to mammals amygdala, being considered a possible substrate for nociception modulation in this animal group. The present study aimed to evaluate the participation of the cannabinoid system of Dm telencephalon on nociception modulation in the fish *Leporinus macrocephalus*. We demonstrated that cannabidiol microinjection in Dm telecephalon inhibits the behavioral nociceptive response to the subcutaneous injection of 3% formaldehyde, and this antinociception is blocked by previous treatment with AM251 microinjection. Furthermore, AM251 microinjection in Dm prior to restraint stress also blockades the stress-induced antinociception. These results reinforce the hypothesis that this pallial telencephalic structure has a pivotal role in nociception modulation in fish.

1. Introduction

A series of recent studies have demonstrated the effect of noxious stimuli on fish behavior and physiology [1–6]. However, knowledge about antinociception and its neurochemical substrates are still incipient. Regarding brain anatomy, fish do not possess a neocortical structure, and the pallium is primitive compared to that of mammals, which has led many scientists to claim the occurrence of complex nociceptive responses but to reject the occurrence of pain perception [7–10]. Despite the absence of neocortical structures, the fish telencephalon possesses structures that are considered homologous to mammal's amygdale and hippocampus; as such, these structures are potentially able to modulate defensive responses. Therefore, the understanding of fish telencephalic mechanisms and neurochemical systems involved in nociception modulation is indispensable to evaluate the degree of complexity of the response to a noxious stimulus and the antinociceptive mechanisms this group.

The teleost fish dorsomedial telencephalon (Dm) is a pallial brain structure that may be a neural substrate for nociception processing, as it has been implicated in memory and a form of learning referred to by Portavela [11,12] as “emotional learning”, and is considered by some authors to be functionally homologous to the mammalian amygdala [11,12]. This homology is also supported by the pattern of connectivity and the presence of $GABA_A$-benzodiazepine receptors [13–17]. Recent research has reinforced this view and related this pallial region to nociception processing, demonstrating that the Dm is also involved in nociception modulation, by inhibiting stress-induced antinociception by microinjection with midazolam, a $GABA_A$ benzodiazepine receptor agonist [18], similar to what is observed after midazolam and diazepam microinjection in the amygdala of mammals [19,20].

Despite the involvement of the Dm GABAergic system in the modulation of fish nociception, there is no evidence of other neurochemical systems involved in this function. Systemic injections of drugs that interfere with cannabinoid and opioid systems modulate fish nociception [5,6], the CB1 receptor gene is highly expressed in the fish dorsal telencephalon [21], and its expression is increased after noxious stimulation in the fish telencephalon [22], suggesting involvement of the telencephalic cannabinoid system in nociception processing.

Thus, the objective of the present study was to evaluate the involvement of the cannabinoid system of the Dm in the induction of antinociception and the modulation of stress-induced antinociception in the fish *Leporinus macrocephalus*, using cannabidiol, a cannabinoid substance, and AM251, a cannabinoid receptor type I (CB1) antagonist.

2. Materials and methods

2.1. Husbandry and set-up

The present study used a total of 112 juvenile piauçu fish (*Leporinus macrocephalus*) (23.45 ± 3.10 g weight), two months old, obtained from a fish farm and maintained in stock tanks (100 × 100 × 60 cm; n = 50) until the experimental period began. Fish were subjected to

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http://dx.doi.org/10.1016/j.physbeh.2017.07.027
Received 7 May 2017; Received in revised form 10 July 2017; Accepted 24 July 2017
Available online 25 July 2017
0031-9384/

unilateral guide cannula implantation in the skull overlying the Dm telencephalon [18], and transferred to individual glass aquaria (40 × 22 × 20 cm, ~18 l) in a closed system with aerated water (pH: 7.35 ± 0.04; temperature: 26 ± 1 °C; unionized ammonia (NH_3): lower than 0.04 mg·l^{-1}), five days prior to the experiment. The sidewalls of the aquaria were covered with opaque white paper and the water was not replaced to avoid disturbance. The light/dark cycle was 12:12 h (light starting at 07:00 and ending at 19:00) and all of the experiments were conducted at the same time of day (between 8:00 and 10:00) to avoid circadian interference. The animals were fed daily with pelleted food corresponding to 3% of the biomass of the fish.

2.2. Surgical procedures

The guide cannula implant was performed using previously described methodology [18]. To implant the guide cannula, the fish were anesthetized through immersion in MS-222 (0.20 g l^{-1}) until the termination of skeletomotor and opercular movements and enveloped in humidified cotton to protect the skin epithelium; during surgery, the fish remained under hydraulic ventilation through the gills with aerated water containing anesthetic maintenance solution (MS-222: 0.10 g l^{-1}).

The animal was attached to a confinement acrylic box coupled with a prior micromanipulator, and a guide cannula with 7 mm in length and 0.5 mm in outer diameter, prepared from a hypodermic needle, was implanted unilaterally (left side) on the skull overlying the Dm telencephalon, following the stereotaxic coordinates of 1.5 mm caudal to frontal zero plane (junction of the olfactory bulb with the telencephalon midline) and 0.35 mm lateral to the midline. The guide cannula was fixed to the skull using a mixture of auto-polymerizing acrylic (Symplex, DFL, Ind. Com) and instant glue (Super Bonder, Loctite).

The guide cannula (7 mm) was inserted in this position, and the injection point, inside the Dm telencephalon, was located at + 0.1 mm, achieved using an injection needle (7.1 mm). After the surgery, all animals recovered from surgery for 5 days.

2.3. Nociceptive test

The nociceptive test was performed as previously described [5,6,18]. Subcutaneous injections of approximately 20 µl of 3% formaldehyde (Formaldehyde P.A. - A.C.S. 37%, pKa = 13.3, stabilized with 10% methanol, Merck, Darmstadt, FRG, www.merck.com) in the region of the adipose fin (located medially between the dorsal and the caudal fin) were used as the noxious stimulus. For subcutaneous injection, fish were removed from the water using nylon nets, wrapped in wet cloth and immediately returned to the water.

2.4. Restraint stress

The restraint procedure was based on a previously described methodology [5,6,18], using a metal screen in the aquarium (30 × 20 × 3 cm) to prevent fish movements for 3 or 5 min without restricting the opercular movements. Restraint is a physiologically stressful condition for fish and inhibits nociceptive responses promoted by the formaldehyde nociceptive test [5,6,18].

2.5. Behavioral analysis

The behavioral analysis evaluated the locomotor activity (distance travelled, 5 min of baseline and 5 min post-stimulus) of fish during the experiment recorded using a camera (Sony CCD-TRV 318, California, USA, www.sony.com) coupled to a computer with image capturing software placed in front of the longest face of the aquarium. The distance travelled was analyzed using EthoVision XT 7.1 software (Noldus Information Technology, Wageningen, NL), and the data are expressed as the differences (Δ) in the values before (baseline) and after (post-stimulus) methodological interventions (Δ = post-stimulus − baseline) (baseline and post-stimulus data are presented as supplementary material). The experimenter was blinded to the treatment during the analysis, and a reliability test was performed for the video analysis.

2.6. Experimental procedures

2.6.1. Experiment 1. Effect of cannabidiol microinjection in the Dm telencephalon on nociceptive response to 3% formaldehyde

In this experiment, the effects of cannabidiol (2-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol, diluted in a solution of saline + 10% DMSO at a concentration of 100 pmol/0.1 µl) microinjection in the Dm telencephalon on the response to noxious stimulation was evaluated. The blockade of this effect was mediated by a previous AM251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide, Tocris Bioscience, Bristol, UK, diluted in a solution of saline + 10% DMSO at a concentration of 50 ng/0.1 µl) microinjection. The cannabidiol and AM251 doses were based on studies in mammals [23]. Fish locomotor activity was recorded for 5 min (baseline) before microinjection of the vehicle or AM251. After 5 min, a group of fish received a cannabidiol microinjection (n = 28; vehicle: n = 14 and AM251: n = 14) and the other group received a vehicle microinjection (n = 28; vehicle: n = 14 and AM251: n = 14, per group). After 5 min, the fish was submitted to the formaldehyde nociceptive test (subcutaneous injection of saline (n = 7 per treatment) or 3% formaldehyde (n = 7 per treatment) and locomotor activity was recorded for 5 min (post-stimulus) (Fig. 1).

2.6.2. Experiment 2. Influence of AM251 microinjection in the Dm telencephalon on the antinociception induced by restraint stress

In this experiment, the effects of AM251 microinjection in the Dm telencephalon on behavioral responses to restraint stress-induced antinociception were assessed. Fish locomotor activity was recorded for 5 min (baseline) prior to the microinjection of 0.1 µl of vehicle or AM251 (50 ng) in the Dm through the previously implanted guide cannula. After 5 min, a group of fish was not subjected to restraint (n = 28; vehicle: n = 14 and midazolam: n = 14), while the other two groups were subjected to 3 or 5 min of restraint (n = 28; vehicle: n = 14 and midazolam: n = 14, per group) to induce antinociception [5,6,18]. After 5 min, the fish were subjected to the formaldehyde nociceptive test (subcutaneous injection of saline or 3% formaldehyde;

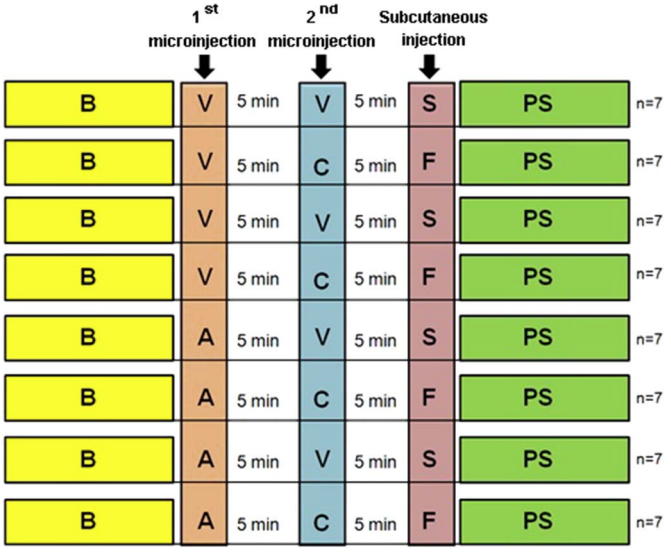

Fig. 1. Schematic drawing of the experimental sequence. B – baseline recording; V – vehicle microinjection; A – AM251 microinjection; C – cannabidiol microinjection; S – subcutaneous saline injection; F – subcutaneous formaldehyde injection; PS – post-stimulus recording.