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Effects of inactivation of serotonergic neurons of the median raphe nucleus on learning and performance of contextual fear conditioning

Karina Genaro Borelli, Ana Carolina Gárgaro, Júlia Maria dos Santos, Marcus Lira Brandão *

Laboratório de Psicobiologia, FFCLRP, Campus USP, Av. Bandeirantes 3900, 14049-901, Ribeirão Preto, SP, Brazil

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Abstract

Several studies have shown that the median raphe nucleus (MRN) is involved in anxiety. However, no study assessed the role of 5-HT mechanisms of MRN in both freezing and fear-potentiated startle (FPS) within a single form of conditioned learning. In this work we examined the effects of neurotoxic lesions of the MRN with NMDA on freezing and FPS of rats submitted to a contextual fear conditioning paradigm, in which they were tested in the same chamber where they received foot-shocks 24 h before. Compared to controls NMDA-injected rats showed a reduction of freezing and FPS in response to contextual cues. Next, we examined the effects of stimulation of 5-HT_{1A} somatodendritic autoreceptors of the MRN with local injections of 8-OH-DPAT either before training or testing sessions conducted 2 or 24 h post-conditioning. Pre-training injections of 8-OH-DPAT intra-MRN reduced both freezing and FPS whereas post-training injections reduced only freezing to the aversive context without changing the FPS. Thus, freezing is easily disrupted by post-training MRN injections of 8-OH-DPAT while memory for FPS remained unchanged. It is proposed that the consolidation of contextual conditioned fear promoting freezing takes place through a slow mechanism of transference of information through 5-HT mechanisms of the MRN-hippocampus pathway. On the other hand, a rapid fear conditioning process operates for FPS, probably through other pathways.

Keywords: Median raphe nucleus; Freezing; Fear conditioning; Startle reflex

Fear conditioning has been reliably evaluated by the amount of freezing and fear-potentiated startle (FPS) that animals display when they return to the context in which they received foot-shocks [3,4,8,15,16]. Many studies have focused on the neural circuits involved in the conditioned fear to contextual (background) or explicit (foreground) stimuli [5,13,15,16]. In this regard, the pathway that ascends within the medial forebrain bundle from the median raphe nucleus (MRN) to hippocampus has received considerable attention [6,7,10]. Electrical stimulation of the MRN causes reduced motor output and several autonomic signs, such as micturition and defecation [9]. 5-HT neurons of the MRN appear to be crucial for the expression of freezing to contextual cues and FPS to light-CS [3,4,5,16]. Indeed, electrolytic or chemical lesions with injections of NMDA (*N*-methyl-D-aspartate), which produce selective destruction of cell bodies, or activation of serotonergic autoreceptors with 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin) of cells of the MRN inhibit contextual freezing conditioning [3,4]. On the other hand, lesions of the MRN impair the acquisition of fear conditioning to explicit cues (light), as assessed by FPS [16]. Injections of the 5-HT_{1A} agonist 8-OH-DPAT into the MRN also increase the locomotor activity [5,11]. However, the effects of this treatment on motor activity and on fear conditioning are dissociated since animals injected with 8-OH-DPAT before conditioning sessions using a tone CS still freeze in testing sessions as much as saline-injected controls [5]. Notwithstanding, no study has examined the role of 5-HT mechanisms of MRN in both freezing and FPS within the contextual conditioned fear as a single form of learning.

The aim of the present study was to examine whether serotonergic mechanisms of the MRN mediate the acquisition of information and expression of contextual conditioned fear. The contextual fear was assessed by different conditioning measurements with opposing motor reactions: the amount of

* Corresponding author. Fax: +55 16 6331609.
E-mail address: mbrandao@usp.br (M.L. Brandão).

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freezing, which interrupts ongoing behavior, and the increase of the startle reflex that the animals display when they return to the context in which they have previously received foot-shocks. This study was conducted on three experiments: (1) analysis of the effects of neurotoxic lesions of the MRN with NMDA on freezing and FPS of rats submitted to a contextual fear conditioning paradigm; (2) analysis of the effects of inactivation of 5-HT neurons with local injections of 8-OH-DPAT on the acquisition of contextual fear; and (3) analysis of the effects of the same treatment on the expression of contextual fear determined 2 or 24 h post-training. It has been demonstrated that MRN injections of the 8-OH-DPAT consistently decreases the neuronal impulse flow by activation of 5-HT_{1A} somatodendritic autoreceptors onto raphe cells [2,3].

Naive male Wistar rats weighing 220–250 g were used. They were housed in groups, under a 12-h-dark:12-h-light cycle (lights on at 07:00 h) at $23 \pm 1\,^{\circ}C$, and given free access to food and water. The experiments were performed in compliance with the recommendations of SBNeC (Brazilian Society of Neuroscience and Behavior), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

For measuring the mean startle amplitude rats were placed into a stabilimeter which consisted of a wire-mesh cage (chamber A, 25 cm × 13 cm × 9 cm) suspended within a PVC frame, which was placed on the response platform. The stabilimeter and platform were located inside a ventilated plywood sound-attenuating chamber (64 cm × 60 cm × 40 cm). The floor of the stabilimeter consisted of six 3.0-mm diameter stainless steel bars spaced 1.5 mm apart. The startle reaction of the rats generated a pressure on the response platform and analog signals were amplified, digitized and analyzed by software (Startle reflex, version 4.10, Med Associates Inc., VT, USA). The presentation and sequencing of the acoustic stimuli were also controlled by the same software and an appropriate interface. A loudspeaker, located 10 cm behind the wire-mesh cage, was used to deliver both the acoustic startle stimuli and a continuous background noise (55 dB SPL). The startle stimulus was a 100 dB, 50 ms burst of white noise, having a rise-decay time of 5 ms. Startle responses were recorded within a time-window of 200 ms after the startle stimulus onset. Calibration procedures were conducted before the experiments to ensure equivalent sensitivities of the response platforms [15,16]. After 5 min of habituation, the rats received a total of 30 startle stimuli at a variable inter-stimulus interval of 30 s on average. The animals were matched into two equivalent groups based on their mean startle amplitude across two sessions in the chamber A (one session per day). Each matching session was 20 min in duration. The mean startle amplitude across the 30 noise bursts on the last matching was taken as baseline condition for comparisons with the startle responses measured later on in the testing condition.

Training: In the contextual conditioned fear procedure used here the animals were conditioned to context in the chamber A (same context group) or chamber B (different context group). The context A consisted of the wire-mesh cage (25 cm × 13 cm × 9 cm) described above. The distinctive characteristics of the context B consisted of a cage of larger dimension (25 cm × 25 cm × 20 cm) with lateral walls made of white walls, ceiling of transparent Plexiglas and floor made of 18 stainless bars with 2.0 mm diameter spaced 1.2 cm apart. The box was located within a ventilated, sound-attenuated chamber (55 cm × 55 cm × 55 cm). In both contexts a loudspeaker, located 10 cm behind the experimental cages, delivered a continuous background noise (55 dB SPL). The animals were placed in the training cage A or B, and 5 min later each rat received 10 CS-US (0.3 mA, 1 s) pairings with a variable inter-trial interval of 60–180 s. The shocks were delivered through the training cage floor by a constant current generator built with a scrambler (Albarsh Instruments, Brazil). Stimulus presentation was controlled by a microprocessor and an I/O board (Insight Equipment, Brazil). Each animal was removed 5 min after the last shock and was returned to its home cage. The duration of the training session was of 30 min.

In the experiments for assessing intra-MRN treatments the animals were anaesthetized with tribromoethanol (250 mg/kg, i.p.) and fixed in a stereotaxic frame (David Kopf, USA). A brain cannula was implanted in the midbrain, aimed at the MRN at an angle of 20° using the following coordinates from bregma: +7.8 mm, antero-posterior; +2.5 mm, medio-lateral; −6.8 mm, dorso-ventral and −2.5 mm, interaural [14]. One week later, the animals were anesthetized again with tribromoethanol (250 mg/kg, i.p., Sigma) and a thin dental needle (o.d. 0.3 mm) was introduced through the guide-cannula until its lower end was 2 mm below the tip of the cannula. The injection needle was linked to a 5 μl Hamilton syringe by means of polyethylene tubing connected to a microinfusion apparatus (Harvard, USA). Neurotoxic lesions were produced by MRN injections of NMDA (Sigma; 6 μg/0.6 μl) 48 h before the training session described above. Infusions were made at 0.1 μl/min and the cannula left in place for a further 2 min, to allow the toxin to diffuse away from the tip. Control groups of animals were similarly anesthetized and injected with physiological saline. Selective evaluation of the involvement of 5-HT neurons was performed with pre- and post-training injections of 8-OH-DPAT (RBI; 1 μg/0.2 μl) into the MRN of awakened animals 10 min before the training or test sessions. Microinjections of the same volume of physiological saline also served as control group.

All testing of the conditioning experiments were conducted in chamber A, which also served as different context for the animals trained in the chamber B. The behavior of the animals was recorded by a video camera (Everfocus) positioned in front of the observation chambers, allowing the discrimination of all possible behavior, with the signal being relayed to a monitor in another room via a closed circuit. The testing sessions for contextual fear conditioning were conducted without presentation of foot shocks. The animals were placed into the startle testing cages and, after 5 min, were pre-