Psychoneuroendocrinology 85 (2017) 69–77

Contents lists available at ScienceDirect

Psychoneuroendocrinology

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

CART neuropeptide modulates the extended amygdalar CeA-vBNST circuit to gate expression of innate fear

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ARTICLE INFO

Keywords:
Innate fear
Fear circuitry
TMT
CART neuropeptide
Amygdala
BNST

ABSTRACT

Innate fear is critical for the survival of animals and is under tight homeostatic control. Deregulation of innate fear processing is thought to underlie pathological phenotypes including, phobias and panic disorders. Although central processing of conditioned fear has been extensively studied, the circuitry and regulatory mechanisms subserving innate fear remain relatively poorly defined.

In this study, we identify cocaine- and amphetamine-regulated transcript (CART) neuropeptide signaling in the central amygdala (CeA) – ventral bed nucleus of stria terminalis (vBNST) axis as a key modulator of innate fear expression. 2,4,5-trimethyl-3-thiazoline (TMT), a component of fox faeces, induces a freezing response whose intensity is regulated by the extent of CART-signaling in the CeA neurons. Abrogation of CART activity in the CeA attenuates the freezing response and reduces activation of vBNST neurons. Conversely, ectopically elevated CART signaling in the CeA potentiates the fear response concomitant with enhanced vBNST activation. We show that local levels of CART signaling modulate the activation of CeA neurons by NMDA receptor-mediated glutamatergic inputs, in turn, regulating activity in the vBNST.

This study identifies the extended amygdalar CeA-vBNST circuit as a CART modulated axis encoding innate fear. CART signaling regulates the glutamatergic excitatory drive in the CeA-vBNST circuit, in turn, gating the expression of the freezing response to TMT.

1. Introduction

Exposure to threatening stimuli evokes a constellation of responses aimed at self-preservation. Genetically ingrained mechanisms engender spontaneous fear, independent of earlier experience, and offer a unique opportunity to dissect an emotion – from its arousal to behavioral end point. While the onset, intensity, persistence and extinction kinetics of these innate responses are tightly regulated, dysregulation of the underlying system may lead to neurological conditions like post-traumatic stress disorders, phobias and panic disorders. Unimodal predator cues, like 2,4,5-trimethyl-3-thiazoline (TMT; an ethologically relevant fear-inducing odorant derived from fox faeces) have been used to delineate the neuroanatomical underpinnings of innate fear (Day et al., 2004; Rosen et al., 2015; Silva et al., 2016; Takahashi, 2014).

TMT is sensed by discrete neurons of the nasal epithelium and Gruenenberg ganglia, which project to the main olfactory bulb as well as the accessory olfactory bulb (Brechbuhl et al., 2013; Kobayakawa et al., 2007; Matsumoto et al., 2010). Downstream to the medial or accessory olfactory bulbs, the TMT generated information is known to transit via the cortical nucleus of the amygdala (CoA) (Root et al., 2014) and medial nucleus of the amygdala (MeA) (Muller and Fendt, 2006). How the information transits from the CoA/MeA to motor output areas like the periaqueductal grey (PAG) remains unclear. A possible route may involve central nucleus of the amygdala (CeA) – ventral bed nucleus of the stria terminalis (vBNST) connectivity that, in turn, communicates to the PAG possibly via specific hypothalamic nodes (Motta et al., 2009; Pagani and Rosen, 2009). A tight coordination between the CeA and BNST is emerging as a major regulatory node in the processing of fear and anxiety in rodents and primates (Fox et al., 2015; Shackman and Fox, 2016). Previous studies based on rodents as well as primates implicate CeA in a variety of fear responses triggered by predator or predator cues (Day et al., 2004; Kalin et al., 2004). Suppression of activity of serotonin-2A receptor expressing neurons of the CeA has been shown to mediate innate fear induced by an artificial TMT-derivative (Isosaka et al., 2015). Exposure to ferret resulted in an increase in the secretion of CRF in the CeA of rat (Merali et al., 2001). Studies from our laboratory and others implicate neuronal activation in the CeA in response to TMT (Butler et al., 2011; Sharma et al., 2014), while silencing of the vBNST by muscimol abolished TMT-induced freezing (Fendt et al., 2003).

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http://dx.doi.org/10.1016/j.psyneuen.2017.08.012
Received 12 March 2017; Received in revised form 7 August 2017; Accepted 9 August 2017

In contrast to conditioned fear, our understanding of the modulatory control of innate fear is limited. The amygdalar circuitry has emerged as a central regulatory hub for conditioned fear. A range of agents, inclusive of fast-acting neurotransmitters like GABA, glutamate, dopamine and serotonin and neuropeptides like corticotropin-releasing factor (CRH), opioid peptides, neuropeptide Y, thyrotropin-releasing hormone, calcitonin gene-related peptide and vasopressin influence fear conditioning (Davis and Whalen, 2001; Schulkin et al., 2005; Shionoya et al., 2013; Spannuth et al., 2011; Tasan et al., 2016). However, little is known about the modulatory processes associated with innate fear. CRF, somatostatin and opioids have been implicated in innate fear processing (Asok et al., 2013, 2016; Figueiredo et al., 2003; Nanda et al., 2008; Roseboom et al., 2007; Wilson and Junor, 2008), and the underlying modes of action and neuroanatomical substrates are just beginning to be understood.

Studies from our laboratory have implicated the neuropeptide CART as an important player in the processing of innate fear within the CeA. Exposure to cat or TMT induced robust freezing in rats, which was dependent on CART signaling (Sharma et al., 2014; Upadhya et al., 2013). In this study, we uncover a CART signaling-sensitive extended amygdalar CeA-vBNST circuitry in TMT-induced fear processing. CART potentiates NMDA-R-dependent excitatory drive in the CeA-vBNST axis and exerts regulatory control on TMT-induced freezing.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats weighing 200–220 g at the time of surgery were used. All the rats were maintained on a 12 h light/dark cycle, at controlled room temperature of 25 ± 2 °C with food and water available ad libitum. The bedding of the cages was changed every week. In order to obviate novelty related stress, all rats were habituated for five days to handling, laboratory conditions and to the test chamber. All experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) constituted by the CPCSEA, Govt. of India.

2.2. Surgery

Stereotaxic surgery and implantation of cannula were carried out according to previously described protocols (Sharma et al., 2014). Briefly, the rats were anaesthetized with intraperitoneal (i.p.) ketamine (60 mg/kg, Aqua Fine Injecta, India) and xylazine (10 mg/kg, Stanex, India) injection. Hair depilator (Anne French, Wyeth, India) was applied to the head to remove hair. Each rat was mounted on the stereotaxic frame with blunt ear bars (Stoelting, USA) and a mid-sagittal incision was made in the scalp to expose the skull. Two stainless steel guide cannulae were implanted bilaterally targeted at the CeA using the stereotaxic coordinates −1.9 mm caudal, ± 4.0 mm lateral and −7.8 mm ventral to the bregma and secured to the skull with anchoring screws and dental cement (DPI-RR cold cure, acrylic powder, Dental Products of India, India). After surgery and between testing, dummy cannulas were inserted into the guide cannulas to prevent occlusion. The animals were placed in separate cages to avoid damage to the guide and dummy cannulae. All rats were allowed one week to recover prior to the start of behavioral testing. Only the rats showing quick recovery and no signs of infection were included in the study. The animals were divided randomly into different groups (n = 6 in each) and habituated to the testing environment for five days.

Post-necropsy, the brain sections were examined for proper placement of the cannulae (Supplementary Fig. S1). The data drawn from the animals with both the cannulae in the target region were considered for analysis.

2.3. Microinjections

For microinjections, the injection cannulae (fabricated in-house; internal diameter 0.16 mm, outer diameter 0.31 mm) connected via PE-10 polyethylene tubing to a microliter syringe (10 µl, Hamilton, USA) and extending 0.5 mm beyond the guide cannulae (fabricated in house as described earlier (Kokare et al., 2011); internal diameter 0.36 mm, outer diameter 0.5 mm) targeting the CeA were used and rats were bilaterally administered different agents according to their treatment group. The control group was bilaterally injected with 0.5 µl of artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 3.35 mM KCl, 1.26 mM $CaCl_2$, 1.15 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 1.2 mM Na_2HPO_4 (pH 7.4)) over a period of 5 min. Similarly, other groups received different treatments such as non-immune serum (NIS; 0.1% bovine serum albumin in aCSF; 0.5 µl/side); CART antibody (1:500 in NIS; 0.5 µl/side; gift from Drs. Lars Thim and Jes Clausen, Novo Nordisk, Denmark); CART peptide (10 ng in 0.25 µl/side; gift from Drs. Lars Thim and Jes Clausen, Novo Nordisk, Denmark); lidocaine hydrochloride (2% solution; AstraZeneca) and MK 801, a non-competitive NMDAR antagonist (5 µg in 0.5 µl/side, diluted in aCSF; Tocris) bilaterally in the CeA. For administration of CART antibody, CART peptide or matching controls in the CeA, the animals were injected 15 min prior to behavioral testing. MK801 and control aCSF were injected 5 min prior to behavior testing, while lidocaine and buffered saline was injected 10 min prior. For double injections, aCSF or MK801 was bilaterally injected into the CeA followed by a second bilateral injection of CART peptide after 5 min. Behavioral tests were conducted 15 min after the second injection.

An additional experiment was conducted to investigate the effect of CART treatment per se on the CeA neurons. Sodium thiopental (60 mg/ml; i.p.) anaesthetized rats were stereotactically injected with CART peptide (10 ng dissolved in 0.5 µl aCSF) bilaterally in the CeA using a 31-gauge needle. Following an interval of 30 min, the animals were perfused transcardially and subjected to immunofluorescence analysis (see below).

2.4. Exposure of rat to TMT and behavior assessment

Behavioral tests were carried out as described earlier (Sharma et al., 2014). In this publication, we characterized the specificity of TMT to induce fear and neuronal activation in the CeA and the vBNST as opposed to butyric acid – a non-specific, aversive response to a noxious odorant. Briefly, rats were habituated to the Plexiglas test chamber having dimensions 8.6 × 8.6 × 20 cm (Wallace and Rosen, 2001) following recovery and equipped with two doors at opposite ends (8.6 × 8.6 cm) each having a 6 × 6 cm opening covered by the filter paper. The animals were habituated for 10 min each day for 5 days. On the 6th day, fifteen minutes after the injections, two filter papers, each coated with 35 µl of TMT were taped over the two openings and the rat was introduced into the test chamber. The behavior of the rat was monitored for a period of 20 min. During the test period, the freezing behavior (absence of all movements except those required for respiration) was recorded and analysed using Noldus Ethovision video tracking system (Netherlands). During the test period, the videos were acquired at 8.33 frames per second. The analysis was conducted by averaging 20 frames. 5% change in body area was set as the threshold to quantify freezing behavior. The data acquisition was by a skilled individual blind to the treatments. The data on freezing are represented as percent of total recorded time.

2.5. Immunohistochemistry

The protocol described in our earlier study was employed (Sharma et al., 2014). Thirty minutes after TMT exposure, the rats were anesthetized (sodium thiopental; 60 mg/kg; i.p.) and perfused transcardially using saline followed by chilled 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). The brains were post-fixed in 4% PFA