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

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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 signalling in the central amygdala (CeA) - ventral bed nucleus of stria terminalis (vBNST) axis as a key modulator of innate fear expression. TMT, a component of fox faeces, induces a freezing response whose intensity is regulated by the extent of CART-signalling 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 signalling 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.

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 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 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 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 Htr2a-positive 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 the increased of CRF at 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).

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 CRH, opioid peptides, neuropeptide Y, TRH, CGRP 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; Asok *et al*, 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 tocat 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.

MATERIALS AND METHODS

Subjects

Adult male Sprague-Dawley rats weighing 200–220 g at the time of surgery were used. All the rats were maintained on a 12 hr light/dark cycle, at controlled room temperature of 25± 2^0 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.

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 (50 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 on the scalp to expose the skull. Two stainless steel guide cannulas 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 cannulas. 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.

Microinjections

For microinjections, the injection cannulas (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 cannulas (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 Dr. Lars Thim, Novo Nordisk, Denmark); CART peptide (10 ng in 0.25 µl/side; gift from Dr. Lars Thim, 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. In another group, aCSF or MK801 was bilaterally injected into the CeA followed by a second bilateral injection of CART peptide after 5 mins. In all the groups, fifteen minutes after the last injection, rats were individually

exposed to TMT odor for 20 min and the behavior recorded and analyzed as described below.

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

Exposure of rat to TMT and behavior assessment

Behavioural tests were carried out as described earlier (Sharma *et al*, 2014). In the latter publication, we characterized the specificity of TMT to induce fear and neuronal activation in the CeA and the vBNST as opposed to 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 mins each day for 5 days. On the 6^{th} 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 (Netherland). The data on freezing is represented as percent of total recorded time.

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) 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 overnight and transferred to 30% sucrose solution at 4^0C for cryoprotection. The brains were serially sectioned on a cryostat in a coronal plane at the 30 µm thickness and stored in 50% glycerol in PBS at 4^0 C. Free- floating sections were rinsed in PBS, incubated in the blocking solution containing monoclonal antibodies against CART (55—102, generous gift by Drs. Lars Thim and JT Clausen, Novo Nordisk, Denmark) diluted in a ratio 1:2000 and polyclonal anti-Fos antibody (1:1000, Santa Cruz, USA) for 24 h in a humid atmosphere at 4^0 C. The sections were then washed and incubated with anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 568 secondary antibodies (A-11001; A-11004; A-11008; A-11011; Invitrogen, Carlsbad, CA) for 2 hr. Further, sections were mounted in a glycerol-based mounting medium (70% glycerol, 0.5% N-propyl-gallate, 20 mM Tris, pH 8.0) containing 4,6-diamidino-2-phenylindole (DAPI; 0.01 mg/ml) and observed under an epifluorescence microscope (AxioImager Z1, Carl Zeiss, Germany) or imaged using a laser scanning confocal microscope (Zeiss LSM 710, Carl Zeiss, Germany). ImageJ was used to adjust the size, contrast, and brightness of the micrographs. Inkscape (ver. 0.91) was used to prepare the panels and diagrammatic representations. In order to ensure reliable comparisons across different groups and maintain stringency in tissue

preparation and staining conditions, all brain sections were processed concurrently under identical conditions.

Morphometric analysis

Morphometric analysis was carried out according to the protocol described in our earlier study (Sharma *et al*, 2014). Briefly, the number of Fos expressing cells were counted from eight sections containing both the sides of vBNST region (A.P -0.12 mm to -0.36 mm with reference to bregma), drawn from each of the six brains in each group. The cell numbers were subjected to Abercrombie's correction to avoid over estimation using the equation $N = (p \times T)/(T + d)$, where N is the corrected cell number, T is the thickness of section, p is the actual profile count and d is the mean nuclear diameter.

Statistical Analysis

Behavior and morphometric data analyses were performed using Mann-Whitney test. All values are expressed as mean ± SEM of the group and differences were considered significant at $p<0.05$. Graphs were plotted using the GraphPad Prism 5.0 statistical software.

RESULTS

CART signalling in the CeA-vBNST circuit modulates expression of TMT-induced innate fear

As the extended amygdalar CeA-BNST circuit has been implicated in processing innate fear (Schulkin *et al*, 2005), we tested if CART signalling modulated the activity of this circuit following exposure to TMT. Induction of Fos in the vBNST was employed as surrogate readout of recent neuronal activation. To test the contribution of CART signalling in processing TMT-induced innate fear responses at the CeA, immunoneutralization of endogenous CART activity was employed. Stereotactic delivery of neutralizing antibody against CART neuropeptide in the CeA of TMT-exposed rat attenuated the freezing response (Fig 1A; $p = 0.0022$; $n = 6$) compared to animals injected with the non-immune serum (NIS). Our previous work had implicated activation of vBNST neurons by TMT (Sharma *et al*, 2014). On similar lines, TMT exposure increased the number of Fos expressing neurons in the vBNST in animals treated with NIS in the CeA. Strikingly, rats injected with CART antibody in the CeA attenuated Fos induction in the vBNST (Fig 1B-F; $p = 0.0043$; $n = 6$). These results, in line with our previous reports (Sharma *et al*, 2014; Upadhya *et al*, 2013), confirm the role of CART in processing innate fear in the CeA.

To directly test if CeA activity regulates innate fear and vBNST activation, we silenced CeA neurons by stereotaxically administering lidocaine in the CeA. Compared to controls, lidocaine injected animals showed reduced freezing in response to TMT (Fig 1G; $p = 0.0022$; $n = 6$). Further, the number of vBNST

cells showing TMT induced Fos activation was also attenuated (Fig 1H; p = 0.0022; n = 6).

These data suggest that CeA mediated activation of vBNST subserves TMT induced fear processing and this circuit may be subject to modulation by CART signaling.

Exogenous CART peptide in the CeA intensifies TMT-induced fear responses and promotes vBNST activation

To test the role of CART signaling in the CeA-vBNST circuit, CART peptide was administered stereotaxically to the CeA. Compared to aCSF treated animals, CART infused rats showed a significantly prolonged freezing response (Fig 2A; p = 0.0087; n = 6). Fos induction in the vBNST was increased in rats treated with exogenous CART peptide compared to those receiving aCSF in the CeA (Fig 2B-F; p = 0.0043; n = 6). These results highlight the importance of CART signaling in fear processing in CeA-vBNST axis.

We also tested if exogenously applied CART peptide in the CeA could directly alter the excitability of the CeA-vBNST axis. Number of Fos-positive neurons increased in both the CeA (Fig 2G; p=0.0043; n=6) and the vBNST (Fig 2H; p = 0.0050; n = 6) when CART peptide was introduced even in the absence of TMT.

The data drawn from CART immunoneutralization, administration of CART peptide (with or without TMT) and silencing of CeA neurons suggest a strong correlation between CART activity in the CeA, activation of vBNST and expression of TMT-induced fear. The strength of CART signalling in the CeA

appears to regulate the intensity of vBNST activation, in turn, gating the expression of innate fear. The intensification of TMT-induced freezing in response to exogenous CART peptide is in line with previous experiments using live cat as the fear-inducing cue (Upadhya *et al*, 2013).

NMDA-R activity in the CeA mediates TMT-induced fear processing

With a view to test the involvement of glutamatergic signaling in the CeA in TMT-induced fear, we blocked the NMDA-R activity with MK801, a non-competitive antagonist of NMDA-R. Administration of MK801 directly into the CeA, attenuated the TMT-induced the freezing response (Fig 3A; $p = 0.0043$; $n = 6$). MK801 treated animals also showed reduced Fos expression in the vBNST compared to aCSF controls (Fig 3B-F; $p = 0.0087$; $n = 6$). The results underscore the role of glutamatergic inputs, acting via NMDA-R, in conveying the fear information over the CeA-vBNST circuit.

CART function in the CeA is mediated by NMDA-R signalling

To test if fear intensification by exogenous CART peptide is mediated by NMDA-R signaling, animals pretreated with MK801 in the CeA were evaluated for fear potentiation by exogenous CART. CART peptide induced increase in freezing, in response to TMT, was attenuated in MK801 pretreated animal (Fig 4A; $p = 0.0043$; $n = 6$). However, vehicle control failed to attenuate the CART peptide augmented response to TMT. Increased expression of Fos in the vBNST, following CART peptide infusion and TMT exposure, was also attenuated upon blocking NMDA-R by MK801 (Fig 4B-F; $p = 0.0022$; $n = 6$).

Taken together, these results suggest that CART activity increases the excitatory drive from CeA to vBNST via potentiation of the NMDA-R activity.

Modulation of vBNST activation by CART signalling in the CeA regulated the intensity of TMT-induced freezing behavior.

DISCUSSION

TMT has been widely used to investigate the processing of innate and unconditioned fear. It serves as a reliable, unimodal and ethologically relevant odorant cue that induces fear in rodents. Fear can be quantified in terms of time the animal shows freezing and other behavioral parameters. In this study, we use TMT to characterize the modulation of innate fear by CART neuropeptide.

CART mediated modulation of the CeA-vBNST axis is central to innate fear

The role of specific amygdalar subregions is well characterized in conditioned fear but not for innate fear. Excitotoxic lesions of the basal (BA) and lateral (LA) nuclei of the amygdala did not affect TMT-induced freezing (Wallace *et al*, 2001). However, muscimol based inactivation revealed the involvement of the basolateral (BLA) and medial amygdala (MeA) (Muller *et al*, 2006) but not of the LA (Fendt *et al*, 2003). The amygdalar cortex (CoA) has also been implicated in fear response to TMT (Root *et al*, 2014). Further downstream, the role of CeA in TMT- and cat-induced fear has been demonstrated in the context of CART neuropeptide signaling (Sharma *et al*, 2014; Upadhya *et al*, 2013).

Elevated expression of Fos protein, c-fos mRNA and egr-1 mRNA in response to TMT was reported in the rat CeA (Asok *et al*, 2013; Sharma *et al*, 2014). These studies also showed increased CART and corticotropin releasing hormone (CRH) expression in the CeA underscoring a functional role of CeA

in TMT-induced fear processing. On similar lines, ferret odor increased CRH and c-fos expression in the rat CeA (Butler *et al*, 2011; Merali *et al*, 2001).

The BNST is a major downstream target of the CeA neurons (Sah *et al*, 2003; Shackman *et al*, 2016). Several studies have implicated the BNST as a central node in processing innate fear. The neurons of the BNST showed Fos induction on exposure of the rat to predator cues, including TMT (Asok *et al*, 2013; Campeau *et al*, 2008; Day *et al*, 2004; Dielenberg *et al*, 2001; Figueiredo *et al*, 2003; Janitzky *et al*, 2009; Masini *et al*, 2005; McGregor *et al*, 2004; Sharma *et al*, 2014). Inactivation of the vBNST by muscimol or norepinephrine antagonists attenuated TMT-induced fear response (Fendt *et al*, 2003; Fendt *et al*, 2005).

The CART peptide is abundantly expressed in the neurons of the CeA while the fiber terminals are seen in the vBNST (Sharma *et al*, 2014; Upadhya *et al*, 2013). In these studies, immunoneutralisation of CART activity in the CeA was used to demonstrate a functional role for CART in processing innate fear. Further, Fos induction was seen in the CeA as well as vBNST in response to TMT (Sharma *et al*, 2014). In the present study, immunoneutralization of endogenous CART at the CeA not only reduced the freezing response to TMT but also attenuate the vBNST activation. To underscore the causality of CART activity in inducing freezing, within the framework of the CeA-vBNST, we show that exogenously administered CART intensifies the behavioral response to TMT and concomitantly augments vBNST activation. Further, silencing of the CeA neurons by lidocaine not only abolished the freezing response to TMT, but also reduced Fos expression in the vBNST. Collectively, the data suggest that the CeA-vBNST axis is central to TMT-

induced fear processing. CART signalling modulates the activity of the CeA neurons, which in turn, regulates vBNST activation and fear expression.

The microscopic organisation of the CART-modulated CeA-vBNST circuit is currently unclear. Given that most CeA-vBNST projections are GABA-ergic (Crestani *et al*, 2013; Dong *et al*, 2001), the occurrence of monosynaptic connectivity appears unlikely to explain the one to one activity correspondence observed in our studies between CeA and vBNST. Disinhibition via an intermediate interneuron is an alternative though this is yet to be experimentally determined. A major limitation in mappping the CART-responsive circuitry is the elusive identity of the CART receptor/s.

The CeA-vBNST circuit identified in this study may be an integral component of information flow from the CoA/MeA to the hypothalamus and PAG. It has been shown that the BNST sends afferents to the PAG passing through the anterior hypothalamic nucleus and ventromedial hypothalamus (Dong and Swanson, 2004, 2006). The CeA may have other parallel outputs including direct afferents to the PAG and the laterodorsal tegmental area, both of which are involved in TMT-induced fear (Kessler *et al*, 2012; Vianna and Brandao, 2003; Yang *et al*, 2016).

CART signaling in the CeA

Our data indicate that CART-signaling modulates the activity of CeA neurons, which, via the vBNST, regulates expression of innate fear. To investigate the mechanism of CART activity in the CeA we investigated the role of NMDA-R-mediated glutamatergic signalling in the CeA neurons. Our results suggest that CART-mediated intensification of the fear response to TMT and

augmented activation of the vBNST, are mediated through NMDA-R activity. Consistent with our observation, in mice, significant proportion of CeA neurons projecting to the vBNST express the NMDA-R in their somato-dendritic compartments (Beckerman and Glass, 2012). Morphine-induced induction of cFos in vBNST neurons is blocked when NMDA-R is genetically silenced in the CeA, implicating functional connectivity between the NMDA-R expressing CeA neurons and the vBNST (Beckerman *et al*, 2012).

It remains unclear whether CART function at the CeA is pre- or postsynaptic. Again, the identification of the CART receptor/s remains a major hurdle in answering this question. CART has been previously found to potentiate NMDA-R activity by promoting phosphorylation of the NR1 subunit in sensory neurons suggesting a postsynaptic function (Chiu *et al*, 2010). In our studies, injection of CART peptide in the CeA was sufficient to increase Fos induction in both the CeA and vBNST suggesting an enhancement of the baseline excitatory glutamatergic drive in the CeA.

Conclusion

The CeA-vBNST axis is emerging as a major neural substrate encoding negative valence in physiological and behavioral responses to stress (Avery *et al*, 2016; Fox *et al*, 2015; Shackman *et al*, 2016). Our study identifies CART signaling as a major modulator of innate fear gating the information flow in the CeA-vBNSTcircuit. These studies define a novel mechanistic framework in our understanding of survival instincts subserved by hard-wired circuitry subject to peptidergic modulation.

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FIGURE LEGENDS

Figure 1 CART signalling in the CeA regulates TMT-induced freezing and activation of the vBNST neurons. The effects of intra-CeA administration of non-immune serum (NIS) or CART antibody (CART Ab) on percent time spent freezing (A) and the number of Fos-positive cells in the vBNST (B) following TMT exposure are represented as means ± SEM. NIS or CART Ab were administered 15 min prior to TMT exposure. Representative micrographs of the vBNST region from NIS (C-D) and CART Ab (E-F) showing Fos staining (C, E) and overlay (D, F) of Fos (green) and DAPI (blue). ac, anterior commissure. Effects of intra-CeA administration of buffered saline or lidocaine on percent time spent freezing (G) and the number of Fos-positive cells in the vBNST (H) following TMT exposure are represented as means ± SEM. The data were analyzed by Mann-Whitney test. N = 6 animals in each group. ** p < 0.01. Scale bar: 50 µm, (C) - (F); 20 µm, insets.

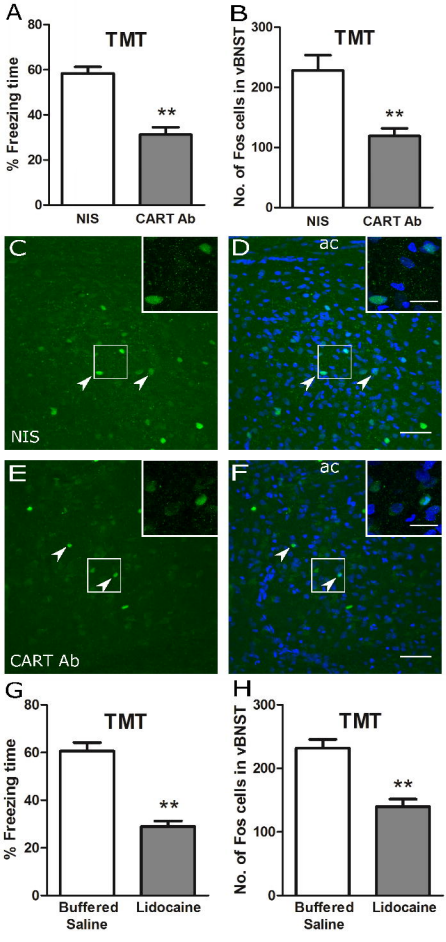
Figure 2 CART peptide intensifies TMT-induced freezing and activation of the vBNST and CeA neurons. The effects of intra-CeA administration of artificial cerebrospinal fluid (aCSF) or CART peptide (CARTp) on percent time spent freezing (A) and the number of Fos-positive cells in the vBNST (B) following TMT exposure are represented as means ± SEM. aCSF or CARTp were administered 15 min prior to TMT exposure. Representative micrographs of the vBNST region from aCSF (C-D) and CARTp (E-F) showing Fos staining (C, E) and overlay (D, F) of Fos (green) and DAPI (blue). ac, anterior commissure. Intra-CeA administration of CARTp in anesthesized rats on the number Fos-positive cells in the CeA (G) and vBNST (H). The data were

analyzed by Mann-Whitney test. *N* = 6 animals in each group (A-B) and *N* = 6 amygdalae in each group (G-H). ** $p < 0.01$. Scale bar: 50 µm, (C) - (F); 20 µm, insets.

Figure 3 NMDA-R signaling in the CeA mediates TMT-induced freezing and activation of the vBNST neurons. The effects of intra-CeA administration of artificial cerebrospinal fluid (aCSF) or MK801 on percent time spent freezing (A) and the number of Fos-positive cells in the vBNST (B) following TMT exposure are represented as means ± SEM. The agents were administered 5 mins prior to TMT exposure. Representative micrographs of the vBNST region from aCSF (C-D) and MK801 (E-F) showing Fos staining (C, E) and overlay (D, F) of Fos (green) and DAPI (blue). ac, anterior commissure. The data were analyzed by Mann-Whitney test. *N* = 6 in each group. ** $p < 0.01$. Scale bar: 50 µm, (C) - (F); 20 µm, insets.

Figure 4 CART activity in the CeA mediates TMT induced freezing and activation of the vBNST neurons via NMDA-R signaling. The effects of intra-CeA administration of artificial cerebrospinal fluid (aCSF) or MK801 followed by CARTp on percent time spent freezing (A) and the number of Fos-positive cells in the vBNST (B) following TMT exposure are represented as means ± SEM. MK801 or aCSF was administered 15 mins prior to CARTp, followed by TMT exposure after another 5 mins. Representative micrographs of the vBNST region from aCSF + CARTp (C-D) and MK801 + CARTp (E-F) showing Fos staining (C, E) and overlay (D, F) of Fos (green) and DAPI

(blue). ac, anterior commissure. The data were analyzed by Mann-Whitney test. $N = 6$ in each group. ** $p < 0.01$. Scale bar: 50 µm, (C) - (F); 20 µm, insets.


A
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% Freezing time
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40
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0
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CART Ab
B
TMT
No. of Fos cells in vBNST
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100
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NIS
CART Ab
C
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D
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E
CART Ab
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G
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80
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No. of Fos cells in vBNST
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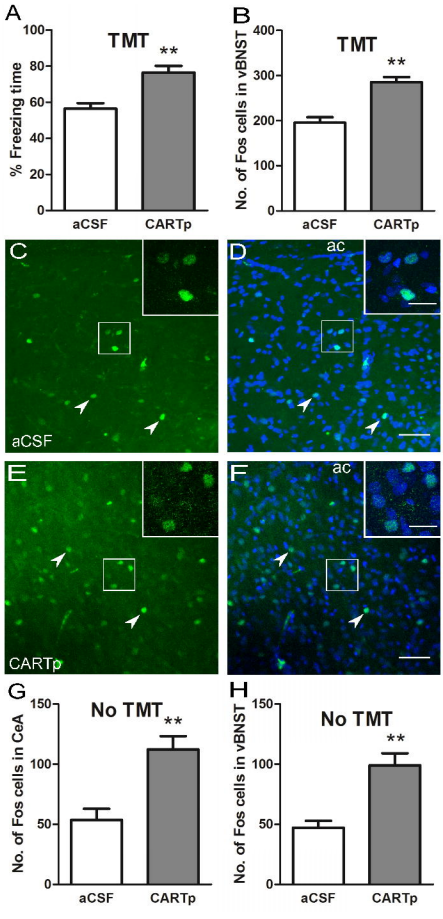
Buffered Saline
Lidocaine

Figure 1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CARTp
F
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No. of Fos cells in vBNST
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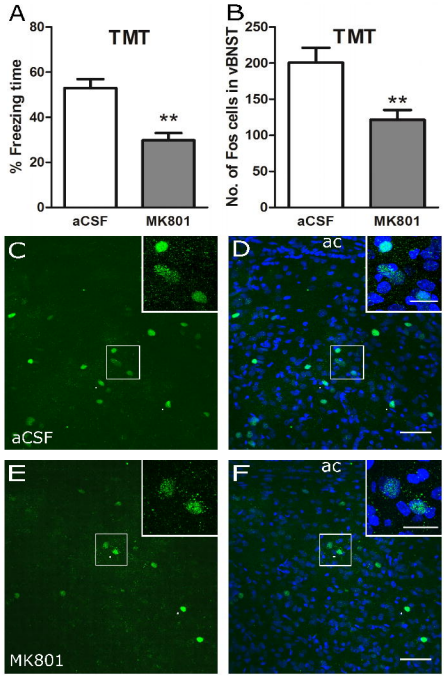
aCSF
CARTp

Figure 2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aCSF
MK801
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No. of Fos cells in vBNST
250
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E
MK801
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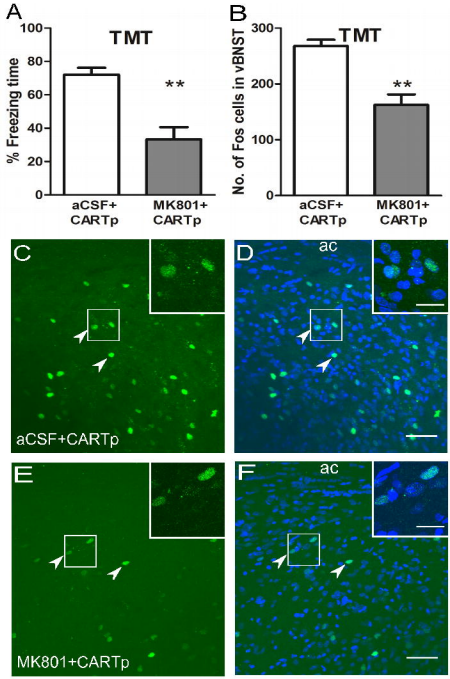
Figure 3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No. of Fos cells in vBNST
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Figure 4