European Neuropsychopharmacology (▮▮▮▮) ▮, ▮▮▮–▮▮▮

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SHORT COMMUNICATION

Knockdown of corticotropin-releasing factor 1 receptors in the ventral tegmental area enhances conditioned fear

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Received 21 January 2016; received in revised form 14 April 2016; accepted 6 June 2016

KEYWORDS
Stress;
Fear;
VTA;
CRF;
Memory

Abstract
The neuropeptide corticotropin-releasing factor (CRF) coordinates the physiological and behavioural responses to stress. CRF receptors are highly expressed in the ventral tegmental area (VTA), an important region for motivated behaviour. Therefore, we examined the role of CRF receptor type 1 (CRFR1) in the VTA in conditioned fear, using a viral-mediated RNA interference approach. Following stereotaxic injection of a lentivirus that contained either shCRF-R1 or a control sequence, mice received tone-footshock pairings. Intra-VTA shCRF-R1 did not affect tone-elicited freezing during conditioning. Once conditioned fear was acquired, however, shCRF-R1 mice consistently showed stronger freezing to the tone even after extinction and reinstatement. These results implicate a novel role of VTA CRF-R1 in conditioned fear, and suggest how stress may modulate aversive learning and memory.

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http://dx.doi.org/10.1016/j.euroneuro.2016.06.002
0924-977X/

Please cite this article as: Chen, N.A., et al., Knockdown of corticotropin-releasing factor 1 receptors in the ventral tegmental area enhances conditioned fear. European Neuropsychopharmacology (2016), http://dx.doi.org/10.1016/j.euroneuro.2016.06.002

1. Introduction

Corticotropin-releasing factor (CRF) is localised in numerous extrahypothalamic brain regions including the ventral tegmental area (VTA), a major structure responsible for motivated behaviour and associative learning (Koob and Heinrichs, 1999; Wise and Morales, 2010). Specifically, CRF signalling within the VTA can influence behaviours related to reward learning, including stress-induced reinstatement of cocaine-seeking (Wang et al., 2005; Blacktop et al., 2011; Chen et al., 2014). The VTA also appears important for aversive learning. For example, VTA dopamine signalling is necessary for the expression of conditioned fear (de Oliveira et al., 2009). However, little is known of the role of CRF in the VTA for conditioned fear. Therefore, we used a lentiviral vector to deliver shRNA targeting CRF receptor 1 (CRFR1) mRNA and examined the effect of CRFR1 knockdown on conditioned fear in mice. This method enables visualisation of the viral transduction in the targeted area, as well as a high level of specificity for CRFR1 over other CRF binding partners. We chose CRFR1 because it is more highly expressed in the VTA and binds CRF with a 10-fold higher affinity than CRF receptor 2 (CRFR2) (Chen et al., 2014; Van Pett et al., 2000).

Stress has diverse effects on learning and memory, and VTA activity can be up- or down-regulated depending on the nature of the stressor (Valenti et al., 2012). If VTA CRFR1 promotes an anxiogenic state that increases the strength of the unconditioned stimulus (US) to promote fear learning, then knockdown of CRFR1 signalling in the VTA may dampen conditioned fear. However, CRFR1 signalling is necessary for stress-induced impairment in memory, and antagonism of CRFR1 reverses memory deficits caused by stress (Urani et al., 2011). Therefore, it is also possible that knockdown of CRFR1 in the VTA may enhance fear learning by alleviating stress. With these two opposing hypotheses in mind, we examined the effects of a viral knockdown of CRFR1 in the VTA on conditioned fear.

2. Experimental procedures

2.1. Animals

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia and approved by the Animal Ethics Committee at the Florey Institute of Neuroscience and Mental Health. In each experiment, naïve adult male C57BL/6J mice (Animal Resource Center, Perth, Australia) were used, and maintained on a reversed 12 h light/dark cycle (lights off: 08:00 with *ad libitum* access to food and water throughout the entire study.

2.2. Surgery

Following 10 days of acclimitisation to the facility, mice were placed under isoflurane anaesthesia (1.5–1.8% in 1 L/min air) with perioperative meloxicam analgesia (3 mg/kg, i.p.), and received bilateral VTA injections (1 μl per side, 0.25 μl/min) of lentivirus with a GFP reporter containing either shRNA targeted against CRFR1 mRNA (shCRFR1) or a control construct containing a scrambled shRNA sequence (shControl virus; Sztainberg et al., 2010; Chen et al., 2014). Stereotaxic coordinates for injections relative to bregma were posterior −3.0 mm; lateral 0.45 mm, and ventral −4.4 mm (Paxinos and Franklin, 2004). Mice were allowed to recover for 3 weeks for optimal viral expression before commencing behavioural studies, and were weighed daily during this period (Sztainberg et al., 2010; Chen et al., 2014).

2.3. qPCR validation of viral knockdown

Adult C57BL/6J mice ($n=8$) were injected with virus and allowed 3 weeks of recovery. Mice were then deeply anaesthetised (80 mg/kg, 0.1 ml/10 g pentobarbitone) before being decapitated. Each brain was immediately placed in a steel brain matrix (model 51386: Stoelting). The brains were sliced 2 mm thick using standard razor blades and frozen over dry ice. The right and left VTA were separately punched out using a 1.2 mm micro dissecting needle, then snap frozen over liquid nitrogen and stored at −80 °C ($n=16$; two separate samples per mouse). RNA was extracted using RNeasy Plus Universal Minikit (Qiagen) and reverse transcribed to generate cDNA using a SuperScript VILO cDNA synthesis kit in accordance with the manufacturers protocol (Invitrogen). Primer sequences used in qPCR reactions were: HPRT1: 5′-GCAGTACAGCCCCAAAATGG-3′ (sense) and 5′-GGTCCTTTTCACCAGCAAGCT-3′ (antisense); CRFR1: 5′TGCCAGGAGATTCTCAACGAA-3′ (sense) and 5′-AAAGCCGAGATGAGGTTCCAG-3′ (antisense); CRFR2: 5′-TACCGAATCGCCCTCATTGT-3′(sense) and 5′-CCACGCGATGTTTCTCAGAAT-3′ (antisense); CRFBP: 5′-GGTCCACGAACCAGGAAATG-3′ (sense) and 5′-ATGCAAGTGTCCGAGGGTAA-3′ (antisense); GFP: 5′-CATGCCCGAAGGCTACGT-3′ (sense) and 5′-CGATGCCCTTCAGCTCGAT-3′ (antisense). qPCR was performed on a ViiA7 real-time PCR system using fluorescent SYBR Green Master Mix (Applied Biosystems). Thermocycler conditions were: 10 min at 95 °C for enzyme activation followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C. Melt curve analysis verified the specificity of amplification products for each primer set. All reactions contained the 4 μl cDNA, 5 μl SYBR Green Master Mix, and 1 μM total primers. Real-time data were normalised to the housekeeping gene HPRT1. GFP mRNA was measured as an indicator of site-specific viral injection.

2.4. Behaviour

2.4.1. Apparatus

Each chamber was equipped with a programmable tone generator and speaker used to deliver auditory cues and a constant-current shock generator to deliver electric shock through the stainless steel grid floor (Med Associates, USA). Chambers were set up in one of two different configurations to act as distinct contexts, as previously described (Handford et al., 2014). All groups were counterbalanced across both contexts. Each session was video recorded and freezing behaviour was measured using a Med Associates Video Freeze system.

2.4.2. Conditioning

In all experiments baseline freezing activity was measured for the initial 2 min of the conditioning session. Mice then received 6 tone-footshock pairings. Each pairing consisted of a 10 s tone (conditioned stimulus; CS, vol: 80 dB; frequency: 5000 Hz) that co-terminated with a 1 s footshock (unconditioned stimulus; US, 0.6 mA). Inter-trial intervals (ITI) ranged from 85 to 135 s with an average of 110 s. Freezing was calculated from the first 9 s of each CS presentation to avoid confounding effects of the shock on movement.

2.4.3. Retrieval/extinction

Retrieval/extinction was carried out the following day in the alternate context to the conditioning session. All mice were first allowed a 2 min period during which baseline freezing was

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