Pharmacology, Biochemistry and Behavior 120 (2014) 1–6

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

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

Blocking of corticotrophin releasing factor receptor-1 during footshock attenuates context fear but not the upregulation of prepro-orexin mRNA in rats

Xiaoyu Chen [a], Sa Li [a], Gilbert J. Kirouac [a,b,*]

[a] Department of Oral Biology, Faculty of Dentistry, University of Manitoba, Winnipeg, Manitoba, Canada
[b] Department of Psychiatry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

ARTICLE INFO

Article history:
Received 21 August 2013
Received in revised form 21 January 2014
Accepted 26 January 2014
Available online 31 January 2014

Keywords:
Orexin
Corticotropin releasing factor
Antalarmin
Footshock
Fear
Stress

ABSTRACT

Hypothalamic neuropeptides called orexins (hypocretins) are well known for their roles in promoting arousal. Orexins have also been shown to play a role in fear and anxiety produced by the exposure of rats to an acute episode of moderately intense footshocks. Recent evidence indicates that stress activates orexin neurons through a corticotropin releasing factor (CRF) mechanism. In this study, we examined the effect of a CRF receptor-1 (CRF-R1) antagonist antalarmin (20 mg/kg, i.p.) given before shock exposure on subsequent expression of contextual fear and the levels of prepro-orexin (ppOX) mRNA in the hypothalamus. Antalarmin decreased fear and ultrasonic vocalization expression to the shock context at 2 and 10 days after shock exposure. However, antalarmin did not prevent the increases in ppOX mRNA produced by the shock experience. This study provides evidence that blocking of CRF-R1 at the time of footshocks attenuates contextual fear. While an increase in the activity of the orexin system may contribute to fear, this activation does not appear to be sufficient for fear expression.

1. Introduction

A population of neurons in the posterior hypothalamus synthesizes peptides called orexins (hypocretins) that have been shown to play an important role in arousal (Carter et al., 2009; Sakurai, 2007). The bioactive orexin-A (OXA) and orexin-B (OXB) peptides, which are produced by the cleaving of prepro-orexin (ppOX), produce their effects by interacting with the orexin-1 receptor (OX1R) and the orexin-2 receptor (OX2R) (de Lecea et al., 1998; Sakurai et al., 1998). Consistent with their role in behavioral arousal, orexin neurons are active during periods of wakefulness, active exploration, and drug seeking (Dayas et al., 2008; Espana et al., 2003; Estabrooke et al., 2001; Harris et al., 2005; Mileykovskiy et al., 2005). In addition, administration of orexins increases locomotor activity and appetitive behaviors (Rodgers et al., 2000; Samson et al., 2010; Thorpe et al., 2005; Thorpe and Kotz, 2005) whereas a loss of orexin signaling leads to narcolepsy, a disorder characterized by a difficulty in maintaining wakefulness (Nishino et al., 2001; Sakurai, 2007).

A number of studies have reported that orexin neurons become active when animals are exposed to a stressful condition (Espana et al., 2003; Furlong et al., 2009; Ida et al., 2000; Winsky-Sommerer et al., 2004; Zhu et al., 2002). There is also good evidence indicating that orexins regulate the physiological (Furlong et al., 2009; Kayaba et al., 2003; Zhang et al., 2010), hormonal (Heydendael et al., 2011) and behavioral responses (Heydendael et al., 2011; Li et al., 2010b) to stress. The neural mechanisms by which stress could lead to activation of orexin neurons are likely to be complex and involve a number of neurochemical signals. One possible candidate for the effect of stress on orexin neurons is corticotropin releasing factor (CRF) which is released by hypothalamic and extrahypothalamic neurons in response to stressful events (Koob and Heinrichs, 1999). Indeed, anatomical studies have shown that CRF-immunoreactive terminals make direct contact with orexin neurons and orexin neurons expressed CRF 1 receptors (CRF-R1) (Winsky-Sommerer et al., 2004). Consistent with this, orexin neurons were shown to be depolarized by CRF via a CRF-R1 whereas stress-induced activation of orexin neurons did not occur in CRF-1R knock-out mice (Winsky-Sommerer et al., 2004).

Previous work from our laboratory demonstrated that ppOX mRNA levels are elevated in rats that had received a brief episode of footshocks 6 or 14 days prior to being sacrificed (Chen et al., in press). In addition, the level of ppOX mRNA was found to be correlated with the amount of immobility displayed by shocked rats placed in the context in which the shocks had been previously given 14 days earlier (Chen et al., in press). In this study, we examined whether systemic injections of the CRF-1R antagonist antalarmin given 30 min prior to a single episode of footshocks attenuated the expression of fear-like immobility and the emission of ultrasonic vocalizations (USV) in the dysphoric range (22 kHz). In addition, we assessed whether blocking of the CRF-R1 attenuated the increases in ppOX mRNA levels.

* Corresponding author at: Department of Oral Biology, Faculty of Dentistry, 780 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W2, Canada. Tel.: +1 204 977 5696; fax: +1 204 789 3913.
E-mail address: Gilbert.Kirouac@umanitoba.ca (G.J. Kirouac).

0091-3057/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pbb.2014.01.013

2. Methods

2.1. Animals

Male Sprague–Dawley rats weighing 130–160 g upon arrival (University of Manitoba, Winnipeg, Manitoba, Canada) were pair-housed in plastics cages (45 cm × 25 cm × 20 cm). They were kept in a colony room on a 12-hour light–dark cycle (lights on 6 am) with controlled temperature (20–24 °C) and humidity (40–70%), and were handled for 5 min on alternate day during a 10 day adaptation period. Rats were given free access to food and water in their home cages. The experimental procedures were in compliance with the Canadian Council on Animal Care and were approved by the Research Ethics Review Board of the University of Manitoba.

2.2. Footshock procedure

Rats were transferred one at a time to a brightly illuminated room (400–500 lx) which was dedicated for the footshock exposure. After a 2 min acclimation period, they received 5 electric footshocks (1.5 mA, 2 s, the interval between shocks ranges from 10 to 50 s randomly presented over 2 min) that were administered by a scrambler via a metal grid (MED Associates, St. Albans, Vermont, USA). The animals were kept in the chamber for an additional 60 s before they were returned to their home cages. The nonshocked rats were exposed to the footshock chamber for the same amount of time but no shocks were delivered. The chamber was cleaned with alcohol (10%) and the bedding under the grid floor was changed after each test.

2.3. Drug injection and behavioral tests

Antalarmin hydrochloride (*N*-butyl-*N*-ethyl-2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine hydrochloride; Cat.# 2778; Tocris, Minneapolis, MN) was dissolved in a solution containing 10% ethanol and 10% cremophor (Sigma–Aldrich, St. Louis, MO). Rats were given antalarmin at a dose of 20 mg/kg (i.p.) or the vehicle 30 min before receiving the footshocks. Fear was measured as the percentage of time that rats were immobile (immobility duration/total exposure time) as defined by a complete lack of body movement except for breathing movements (Fanselow, 1980). The number of USV emitted during this period was also recorded (product number ANL-937-1A-R; Med Associates) and later analyzed for the presence of calls within the dysphoric range (20–30 kHz).

Fear expression to the footshock chamber was evaluated in all rats at 2 and 10 days after the shock chamber exposure day. In this case, rats were placed in the footshock chamber for 5 min (no shocks were given) and the amount of immobility and the number of USV were quantified as described above. Fourteen days after the shock exposure, rats were anaesthetized with chloral hydrate (600 mg/kg, i.p.), perfused with 200 ml ice-cold 0.1 M phosphate buffered saline (PBS; pH 7.4), brains removed and frozen with dry ice, and stored at −80 °C. Coronal 20 μm sections of the posterior hypothalamus were made using a cryostat and mounted on slides that were stored at −80 °C until the hybridization procedure was done.

2.4. In situ hybridization

Sections of the hypothalamus containing a large numbers of orexin neurons were selected (approximately 3.0 posterior to bregma) based on immunohistochemical staining for orexin-A using the series of sections (Chen et al., in press). All hybridization solutions were treated with 1% diethylpyrocarbonate and autoclaved to inhibit RNase activity. Frozen brain sections were fixed with ice-cold 4% paraformaldehyde (pH 7.4) for 10 min. The sections were acetylated in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 min, washed with 2× sodium chloride-sodium phosphate-EDTA buffer (2 × SSC; 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 10 min, and dehydrated with 50%, 70%, 95% and 100% ethanol (1 min each). Subsequently, the sections were delipidated in chloroform solution for 8 min and washed with 100% ethanol followed by rehydration in 95% and 80% ethanol (1 min each). A 36-mer synthetic oligonucleotide probe (TTC GTA GAG ACG GCA GGA ACA CGT CTT CTG GCG ACA; complementary to bases 115–150 of rat ppOX) (Yamamoto et al., 1999) was labeled at the 3′-end with ^{32}P-dATP (BLU512A250UC, Perkin Elmer, Waltham, MA, USA) to a specific activity of >12 TBq/mmol by using terminal deoxynucleotideyl transferase (Cat. # M1871, Promega, Madison, WI, USA). The probe for ppOX mRNA is not homologous to any other known sequences as shown using homology screening of Genebank/EMBL sequences. The labeling reactions were done in a 37 °C water bath for 90 min and the radio-labelled probe was purified using illustra Probe Quant G-50 Micro Columns (28-9034-08, GE Healthcare, Buckinghamshire, UK). The purified probe was mixed with Ultrahyb-oligo hybridization buffer (AM8663, Applied Biosystems, Frederick, MD, USA), heated to 70 °C for 10 min, and then cooled on ice. The final concentration of the probe in the hybridization buffer was in a range of 1.8–2.2 pmol/ml and had a specific activity of 10^7 cpm/ml. Hybridization was done by covering the sections with 100 μl of hybridization mixture and incubating them overnight at 38 °C. The negative control experiments were done by adding 100-fold excess of the unlabeled probe to the sections. The slides were rinsed in 1× SSC and post-washed four times in 50% formamide in 2 × SSC solutions for 30 min at room temperature followed by post-wash in 1 × SSC solution for 1 h repeated 3 times. After dehydration with graded ethanol, the slides along with autoradiographic ^{14}C microscale standard strip (31–883 nCi/g) were exposed to Amersham hyperfilm (GE Healthcare, Buckinghamshire, UK) for 12 days in cassettes. The hyperfilms were developed with Kodak GBX developer and fixer (Sigma–Aldrich, St. Louis, MO, USA). Autoradiographic images of individual sections were photographed with an Olympus BX51microscope equipped with a digital camera (SPOT RT Slicer, Diagnostic Instruments Inc, Sterling Heights, MI, USA). The medial and lateral parts of the orexin population were sampled manually by making a rectangular selection bilaterally on the region of interest (analyzed by NIH Image-J software). The optical density of an area of the film outside the orexin population was subtracted from the optical density of the orexin population. The mean optical density of each region was interpolated along the calibration curve derived from the optical density of ^{14}C microscale standards.

2.5. Statistics

A two-way ANOVA was done to investigate the main and interaction effects of “shock” (nonshocked or shocked rats) and “antalarmin” (vehicle or antalarmin treated) on fear responses and ppOX mRNA levels. This was followed by pair-wise comparisons to assess differences between individual groups. Data analyses were performed using SPSS software, version 19.0. A value of $p < 0.05$ was considered to be significant and the data are presented as mean ± SEM.

3. Results

3.1. Effect of antalarmin on the immediate response to footshocks

The two-way ANOVA revealed a significant main effect for “shock” on immobility ($F_{(1,35)} = 518.75$, $p < 0.001$; Fig. 1A) and USV calls ($F_{(1,35)} = 107.17$, $p < 0.001$; Fig. 1A). Antalarmin had no effect on immobility or the number of USV calls emitted nor was there an interaction between “shock” and “antalarmin”. The pairwise comparisons indicated that shocked rats treated with the vehicle ($p < 0.001$) and antalarmin ($p < 0.001$) had exhibited more immobility than nonshocked rats. Likewise, shocked rats treated with the vehicle ($p < 0.001$) and antalarmin ($p < 0.001$) emitted more USV than nonshocked rats. The two-way ANOVA revealed a significant effect