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Both Ox1r and Ox2r orexin receptors contribute to the cardiovascular and locomotor components of the novelty stress response in the rat

Mirza I. Beig, Bruno W. Dampney, Pascal Carrive*

School of Medical Sciences, the University of New South Wales, New South Wales, NSW 2035, Australia

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ABSTRACT

Orexin contributes to the expression of the cardiovascular and behavioural response of some forms of stress, including novelty stress. Thus, Almorexant, a dual receptor antagonist that blocks the two known orexin receptors, Ox1R and Ox2R, reduces these responses. However, it is not known if the reduction results from blockade of one receptor only or both. To answer this question, the selective Ox1R antagonist ACT335827 and the selective Ox2R antagonist EMPA were injected intragastrically (300 mg/kg) or intraperitoneally (30 and 100 mg/kg) either alone or as a cocktail and compared to Almorexant in rats exposed to novelty stress. Cardiovascular and locomotor responses were recorded by radio-telemetry. Triple immunolabelling was also conducted to establish the distribution of Ox1R and Ox2R in sympathetic preganglionic neurons and orexin neurons. Intraperitoneal injections of ACT335827 (100 mg/kg) reduced the pressor and tachycardic but not the locomotor response of novelty (by 32% and 48%, respectively). Intraperitoneal injections of EMPA (100 mg/kg) only reduced the pressor response (42%). However when given together, ACT335827 and EMPA reduced all 3 components (65%, 60% and 57% of the tachycardic, pressor and locomotor responses, respectively) as Almorexant (100 mg/kg) did (69%, 87% and 72%, respectively). Triple immunolabelling revealed that sympathetic preganglionic neurons were mainly Ox1R positive only while orexin neurons were both Ox1R and Ox2R positive. This study shows that orexin's contribution to the cardiovascular and locomotor components of the novelty stress response is not mediated by one receptor alone, but by both receptors and at different levels of the neuraxis.

1. Introduction

It is now well established that orexin A and orexin B, two neuropeptides originating from neurons in the dorsal tuberal hypothalamus, play a major role in the control of arousal, from maintenance of wakefulness to the expression of motivated behaviour (Berridge et al., 2010; Boutrel et al., 2010; Chase, 2013; Sakurai and Mieda, 2011). Integration of the many components of arousal, including cortical arousal, cardiorespiratory activation and motor activity is achieved through the widespread connections that these orexinergic neurons make throughout the brain and spinal cord (Date et al., 2000, 1999; Kerman et al., 2007; Krout et al., 2005; Llewellyn-Smith et al., 2003; Peyron et al., 1998; van den Pol, 1999). Orexin mediates its effects via two G protein coupled receptors, Ox1R and Ox2R, which are both excitatory (Gotter et al., 2012; Sakurai et al., 1998; Scammell and Winrow, 2011; van den Pol et al., 1998) and have partly overlapping distributions in the brain (Bäckberg et al., 2002; Cluderay et al., 2002; Hervieu et al., 2001; Lu et al., 2000; Marcus et al., 2001). The exact role of the two receptors is not clear, but their existence and different distributions raise the possibility of separate contributions to the different components of arousal. This could be very important from a therapeutic point of view.

Much work has already been done on the contributions of Ox1R and Ox2R to the cortical arousal component of orexin's action. Natural mutation or knock out of the Ox2R gene produces symptoms similar to narcolepsy; therefore attention has focused on Ox2R as the main receptor for the maintenance and regulation of cortical arousal (Gotter et al., 2012; Lin et al., 1999; Willie et al., 2003). Pharmacological studies with selective Ox2R agonists and

Abbreviations: ChAT, Choline acetyl transferase; ΔHR, relative change in heart rate; ΔMAP, relative change in mean blood pressure; DORA, dual orexin receptor antagonist; HR, heart rate; MAP, mean blood pressure; Ox1R, orexin receptor 1; Ox2R, orexin receptor 2; OxA, orexin A; SORA, selective orexin receptor antagonist; SPN, sympathetic preganglionic neuron.

* Corresponding author. Tel.: +61 2 9385 2467; fax: +61 2 9385 0023.
E-mail address: p.carrive@unsw.edu.au (P. Carrive).

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antagonists initially lent support to this view (Akanmu and Honda, 2005; Dugovic et al., 2009; Gozzi et al., 2011), however recent studies comparing Ox1R and Ox2R knock-out mice or selective antagonists of the two receptors in rats indicate that both receptors contribute to cortical arousal (Mieda et al., 2011; Morairty et al., 2012).

Less is known about Ox1R and Ox2R contributions to the cardiovascular and locomotor components of orexin's action. We and others have shown that orexin contributes to the cardiovascular and behavioural components of some forms of stress responses such as psychosocial stress, conditioned fear and novelty (Furlong et al., 2009; Kayaba et al., 2003; Kuwaki et al., 2008). In particular, we have shown that the dual orexin receptor antagonist (DORA) Almorexant markedly reduces the cardiovascular and locomotor components of the novelty stress response (Furlong et al., 2009). However, it is not clear if this effect resulted from blockade of one receptor only or of both Ox1R and Ox2R, as has been shown for cortical arousal. To answer this question, in the present study we compare the effect of Almorexant to two selective orexin receptor antagonists (SORA), the Ox1R SORA ACT335827 (Steiner et al., 2013a) and the Ox2R SORA EMPA (Malherbe et al., 2009a). The drugs were administered systemically.

In separate but complementary anatomical experiments, we used triple immunolabelling to investigate the distribution and colocalization of Ox1R and Ox2R in two groups of neurons which we know have orexin receptors and are necessary for the expression of the cardiovascular and/or locomotor response of novelty stress: sympathetic preganglionic neurons (SPN) and the orexin neurons themselves. We reasoned that these experiments could reveal some of the sites of actions of DORAs and that segregation or colocalization of the receptors could provide important clues regarding their mode of action.

2. Materials and methods

2.1. Animals

The study was performed on male Wistar rats (275–300 g) purchased from the Animal Resource Center, Perth, Australia. The animals were housed in individual home cages (65 × 40 × 22 cm) with *ad-libitum* food and water on a normal 12 h light/dark cycle (lights off at 19:00). All efforts were made to reduce animal pain or discomfort. All the procedures were approved by the Animal Ethics Committee of the University of New South Wales and complied with the rules and guidelines on animal experimentation in Australia. All efforts were made to minimise animal suffering and reduce the number of animals used.

2.2. Implantation of radio-telemetric transmitters

Surgery was conducted under isoflurane anaesthesia (1.5% in 100% oxygen) after administration of the analgesic Carprofen (5 mg/kg, Pfizer, Australia), the antibiotic Benacillin (0.2 ml, Troy labs, Australia) and Atropine (60 μg/kg, Pfizer, Australia). The level of anaesthesia was assessed from the corneal or pinch reflex every 10 min and the isoflurane concentration adjusted accordingly. The rats were implanted with radio-telemetric transmitters (TA11PA-C40, Data Sciences International, Saint Paul, MN, USA) for recording of arterial pressure as previously described (Beig et al., 2007). In brief, the catheter of the pressure transmitter was inserted in the femoral artery and pushed into the abdominal aorta. The transmitter was placed in the peritoneal cavity. Rats were allowed to recover for at least 1 week after surgery. During this period they were handled every day to habituate to the experimenter.

2.3. Drugs

The DORA Almorexant and the Ox1R SORA ACT335827 were gifts from Actelion Pharmaceuticals. The Ox2R SORA EMPA was a gift from Roche. Almorexant and ACT335827 were injected intragastrically and intraperitoneally. EMPA was injected intraperitoneally only as it was not available in sufficient quantities to be injected intragastrically. The intragastric dose of Almorexant and ACT335827 was 300 mg/kg as in Furlong et al. (2009) and Steiner et al. (2013a). They were dissolved in 0.25% methyl-cellulose (Methocel®, Sigma Aldrich, Australia) in a 5 ml/kg volume. Intragastric administration was done with a flexible plastic feeding needle (Instech Solomon, Plymouth Meeting, PA, USA). For intraperitoneal injections, two doses were tested: 30 mg/kg and 100 mg/kg as in Morairty et al. (2012), The drugs were dissolved in either 20% Cyclodextrin (Sigma Aldrich, Australia) for Almorexant, normal saline for ACT335827 or 0.3% Tween 80 (Sigma Aldrich, Australia) for EMPA. The volumes of injection were 0.7 ml for the low doses and 1.0–1.5 ml for the high doses. The solutions were prepared on the day of the experiment.

2.4. Testing procedure for novelty stress and experimental design

On the day of the experiment the telemetric transmitter was switched on and baseline data were acquired for at least 2 h while the animal was at rest in its home box. The drug or its vehicle was then administered and the animal returned to its home cage. Two and a half hours later, the animal was gently placed in a clean box with no bedding for 30 min. The box had no lid but its four sides were extended with 60 cm high plexiglass walls. The animal was then returned to its home box and recording continued for at least 1 h. Each animal tested with a drug at a given dose was also tested with the corresponding vehicle in a counterbalanced order. Animals could be tested for the effects of up to three different drugs or two doses of the same drugs. When this was the case, the order of the drug or of the dose was randomised. At least 48 h separated two consecutive tests.

2.5. Data collection and analysis

Three parameters were extracted from the telemetric probe signal: heart rate (HR), mean arterial pressure (MAP) and body movement (Activity, expressed in arbitrary units, a.u.) (see Furlong et al. (2009)). The parameters were later averaged over 1 min periods.

Drug effects were analysed using Prism 6 (GraphPad Software, Inc). For each drug and dose we compared drug injected and vehicle injected animals using a two way repeated measure ANOVA where time was the repeated measure and drug the independent factor. We only report the main drug effect. Three periods were analysed: the 20 min period preceding the injection, the 20 min period preceding the test and the 30 min period covering the novelty test. Figs. 1–6 show the time course of the effect minute by minute, expressed as absolute values (HR, MAP, Activity) and as values relative to the 20 min pre-test baseline (ΔHR,ΔMAP). To allow comparisons of the effects across drugs, areas under the curve of ΔHR, ΔMAP and Activity were expressed as a percentage of the vehicle control averages (Fig 7).

2.6. Triple immunolabelling

Three male Wistar rats were killed with an overdose of pentobarbitone and perfused transcardially with 4% paraformaldehyde after a brief saline wash. The brain and portions of the thoracic cord (T_2–T_{10}) were removed, cryoprotected in 20% sucrose for 2 days and cut into three series of 30 μm thick transverse sections with a cryostat. Two series were processed. They were first incubated in 0.1M citrate buffer pH 6.0 at 72 °C for 30 min for antigen retrieval (see Shi et al., 1995) and then washed in 50% alcohol and 5% normal horse serum (30 min each). Both series were incubated with a chicken IgY anti-Ox1R (1:50, T-1524, BMA Biomedicals, Switzerland, directed against a portion of the second extracellular loop of the human/rat/porcine Ox1R) and a goat IgG anti-Ox2R (1:50, sc8074 Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA directed against a region near the C-terminus of the human Ox2R). Both antibodies were affinity purified polyclonal antibody designed for immunohistochemistry. A third antibody was added to this cocktail of primary antibodies. In one series it was a rabbit IgG anti-Choline Acetyl Transferase (ChAT, 1:250, R-043-100, Biosensis, Australia) to reveal motoneurons. In the other series it was a mouse IgG anti-orexin A (OxA, 1:4000, MAB763, R&D Systems, Minneapolis, MN, USA) to reveal orexin neurons. This was followed two days later by a 2 h incubation in a cocktail of secondary antibodies containing an Alexa Fluor® 488 donkey anti-chicken IgY (1:200, Jackson Immunoresearch, West Grove, PA, USA green) to reveal Ox1R, an Alexa Fluor® 594 donkey anti-goat IgG (1:200, Jackson Immunoresearch, red), to reveal Ox2R and an Alexa Fluor® 350 donkey anti-rabbit or anti-mouse IgG (1:200, Invitrogen, Australia, blue) to reveal ChAT or OxA respectively. The sections were mounted, dried and coverslipped with ProLong® Gold Antifade (Life Technologies, Australia). Photography and analysis was done with the Stereo-Investigator software (MBF bioscience). Only OxA labelled neurons in the hypothalamus and ChAT labelled neurons in the intermediolateral column (IML) of the thoracic cord were analysed. OxA and ChAT cells that were single or double and triple labelled with Ox1R and Ox2R were plotted and counted on representative sections. The counts were expressed relative to the total number of OxA or ChAT neurons and then averaged across the 3 brains.

3. Results

3.1. Pharmacological experiments

Figs. 1–6 show the entire time course of the effects of each drug and its vehicle, starting 30 min before the injection (intragastric or intraperitoneal) until 60 min after the end of the novelty stress test. HR and MAP changes appear on the left of the figures as absolute values. On the right, they are shown relative to their pre-test baselines (ΔHR and ΔMAP). The average values of ΔHR, ΔMAP