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Distribution of the orexin-1 receptor (OX_1R) in the mouse forebrain and rostral brainstem: A characterisation of OX_1R-eGFP mice

Sarah Sulaiman Ch'ng, Andrew J. Lawrence *

Florey Institute of Neuroscience and Mental Health, Melbourne Brain Centre, University of Melbourne, Parkville, VIC 3010, Australia

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ABSTRACT

We have utilised a transgenic reporter mouse in which green fluorescent protein (GFP) expression is driven by the orexin-1 receptor (OX_1R) promoter to systematically map the distribution of OX_1R-expressing neurons throughout the mouse forebrain and rostral brainstem. GFP labelling was observed in perikarya and fibres in an extensive range of brain loci encompassing the olfactory and cerebral cortices, dorsal and ventral pallidum, hippocampus, amygdaloid regions, septal areas, thalamic nuclei, hypothalamic nuclei and several brainstem regions, consistent with previous studies of OX_1R mRNA expression. This is the first study to systematically characterise the neuroanatomical distribution of OX_1R in the OX_1R-eGFP mouse, confirming its veracity as a faithful reporter of OX_1R expression and utility for future studies assessing the role of OX_1R in more complex behaviours.

Introduction

The neuropeptides orexin A and orexin B are produced by alternative splicing from the same prepro-orexin precursor (de Lecea et al., 1998; Sakurai et al., 1998). Orexins are only expressed in neurons within the lateral hypothalamus (LH), perifornical area (PFA) and dorsomedial hypothalamus (DMH) (de Lecea et al., 1998; Sakurai et al., 1998). Orexin-expressing neurons have highly divergent projections throughout the entire neuraxis (Peyron et al., 1998), supporting a role for orexin in regulating various physiological processes such as sleep/wakefulness, arousal, feeding behaviour, energy metabolism, hormone secretion and autonomic homeostasis (Sakurai et al., 1998; Sutcliffe and de Lecea, 2000; Siegel, 2004; Meister, 2007; Berthoud and Munzberg, 2011). Additionally, considerable evidence implicates the orexin system in reward processing and drug-seeking behaviour (Boutrel et al., 2005; Harris et al., 2005; Lawrence et al., 2006; Mahler et al., 2012; Baimel et al., 2015).

Orexins act on the orexin-1 (OX_1R) and orexin-2 receptor (OX_2R) which are G-protein coupled receptors (GPCRs). Although it was initially believed that OX_1R coupled exclusively to G_q and OX_2R to either G_q or $G_{i/o}$, more recent evidence has demonstrated that both receptors can couple to several different G-proteins (Kukkonen and Leonard, 2014). In accordance with the diffuse projections of the orexin neurons, OX_1R and OX_2R are widely expressed throughout the brain in both distinct and overlapping patterns (Trivedi et al., 1998; Marcus et al., 2001). OX_1R has been identified in cortical regions including the prefrontal and infralimbic cortices, the bed nucleus of the stria terminalis (BNST) and brainstem regions such as the dorsal raphe (DR) and locus coeruleus (LC). In contrast, OX_2R is more densely expressed throughout the cortex, in the nucleus accumbens (NAc), medial thalamus and various hypothalamic nuclei. Both receptors are moderately expressed in the ventral tegmental area (VTA) (Trivedi et al., 1998; Marcus et al., 2001; Gozzi et al., 2011). This pattern of distribution implies a possible functional divergence between the two receptors. Indeed, much of the evidence to date points to a general functional dichotomy between orexin receptor function whereby OX_2R signalling is associated with arousal and OX_1R signalling with reward-seeking (Aston-Jones et al., 2010; Mahler et al., 2012). Nevertheless, there is emerging evidence that the functions of the two receptors are not strictly mutually exclusive. For example, despite the established role of

Abbreviations: BNST, bed nucleus of the stria terminalis; DAB, 3,3′ diaminobenzidine tetrahydrochloride; DMH, dorsomedial hypothalamus; DR, dorsal raphe; eGFP, enhanced green fluorescent protein; EPSC, excitatory post-synaptic current; GPCR, G-protein coupled receptor; IR, immunoreactivity; LH, lateral hypothalamus; mRNA, messenger ribonucleic acid; NAc, nucleus accumbens; nu, nucleus; OX_1R, orexin-1 receptor; PBS, phosphate buffered saline; PFA, perifornical area; PFC, prefrontal cortex; PVN, paraventricular hypothalamus; PVT, paraventricular thalamic nucleus; RT, room temperature; SCN, suprachiasmatic nucleus; VMH, ventromedial hypothalamus.

* Corresponding author. Tel.: +61 3 9035 6692; fax: +61 3 9035 3107.
E-mail address: andrew.lawrence@florey.edu.au (A.J. Lawrence).

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OX_1R in reward-seeking, we have recently demonstrated that selective antagonism of OX_2R attenuates self-administration of alcohol (but not cue-induced alcohol-seeking) in alcohol-preferring rats (Brown et al., 2013). Additionally, ethanol consumption is associated with an increase in OX_2R mRNA but not OX_1R mRNA in the anterior paraventricular thalamic nucleus (aPVT) and local antagonism of OX_2R in the aPVT reduces ethanol intake (Barson et al., 2014). As such, this suggests that OX_1R and OX_2R possess partially overlapping and/or complementary functions in behavioural modulation.

Several studies have mapped the distribution of OX_1R messenger ribonucleic acid (mRNA) expression in the rodent brain (Trivedi et al., 1998; Marcus et al., 2001). However, there is a paucity of studies examining OX_1R expression using immunohistochemical techniques due to the notorious difficulty in generating robust antibodies to GPCRs. To overcome this issue, we have taken advantage of a recently generated line of transgenic mice in which enhanced green fluorescent protein (eGFP) expression is driven by the OX_1R promoter to fully characterise the distribution of OX_1R in the mouse brain. Recently, we have utilised this mouse to assess the distribution of OX_1R in the mouse brain stem and pons and delineate the neurochemical phenotype of OX_1R-expressing neurons within the mouse hindbrain (Darwinkel et al., 2014). However, a comprehensive neuroanatomical map of OX_1R expression throughout the forebrain of this mouse has yet to be performed. The aim of the present study was thus to systematically characterise the distribution of OX_1R throughout the mouse forebrain and rostral brainstem based on GFP immunoreactivity (IR) and thereby confirm the fidelity of this mouse as a valid reporter for OX_1R expression.

Materials and methods

Mice

The following experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986, under the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. All experiments were also approved by the Florey Animal Ethics Committee. To systematically map the distribution of OX_1R within the mouse brain, OX_1R-eGFP mice bred on a CD-1 background ($n = 7$) from the Mutant Mouse Regional Resource Centre were used. In these mice, a GFP reporter gene followed by a polyadenylation sequence was inserted into BAC clone RP23-282L14 at the start codon of the first coding exon of the Hcrtr1 gene. The modified BAC construct was injected into the pronuclei of FVB/N fertilised oocytes. Hemizygous offspring were mated to Crl:CD1(ICR) mice. The founder mice were obtained from Dr Danny Winder (Vanderbilt University, USA) *via* Dr Paul Kenny (Mt Sinai School of Medicine, USA). The genotypes of the experimental mice were confirmed using polymerase chain reaction (PCR). Three wildtype mice were used as controls. All mice used in the study were males between the ages of 10–12 weeks. Mice had *ad libitum* access to tap water and standard laboratory mouse chow under a constant 12-h reverse light-dark cycle (dark 0700–1900 h).

Genotype determination

DNA extraction

A tail sample was removed from each mouse for DNA collection and incubated with 100 μL proteinase K solution and 25 μL tissue preparation solution for 20 min at room temperature (RT). Samples were then heated at 95 °C for 3 min after which 100 μL neutralisation solution was added to each tube. All DNA extraction reagents were obtained from Sigma–Aldrich, St Louis, MO, USA.

Polymerase chain reaction (PCR)

A 1 μL aliquot of each extracted DNA solution was added to 11.3 μL dH_2O, 0.5 μL of Hcrtr1 (30803) F1 primer (sequence 5′-CTC CTT TCT TCC CTC CTT CCT TTC TT-3′), 0.5 μL GFP R2 primer (sequence 5′-TAG CGG CTG AAG CAC TGC A-3′) and 12.7 μL GoTaq solution. The samples were placed in a thermocycler and heated for 5 min at 94 °C before 40 cycles consisting of 15 s (94 °C), 30 s (65–55 °C, with a decrease of 1 °C per cycle for the first 10 cycles) and 40 s (72 °C) were commenced. This was followed by an amplification step for 5 min at 72 °C. Samples were then cooled and held at 4 °C. The conditions used were adapted from the protocol developed by Mutant Mouse Regional Resource Centre (University of California, Davis) (http://www.mmrrc.org/).

Gel electrophoresis

PCR products were visualised by standard gel electrophoresis using 1.5% agarose gel with 0.02% SYBR Safe DNA gel stain (Invitrogen, CA, USA). The gels were run at a constant voltage of 100 V for 30 min. A 1 kb DNA reference ladder (Bioline, London, UK) was loaded into the first lane of the gel to serve as a molecular weight marker. Additionally, dH_2O was loaded into the last lane of the gel to serve as a negative control.

Tissue collection and preparation

Mice were anaesthetised with intraperitoneal injection of sodium pentobarbitone (80 mg/kg) and transcardially perfused with 50 mL of phosphate buffered saline (PBS 0.1 M, pH 7.4) followed by fixation with 100 mL of 4% paraformaldehyde in 0.1 M PBS. The mice were subsequently decapitated, their brains excised and post-fixed in 20% sucrose/4% paraformaldehyde overnight at 4 °C before being frozen over liquid nitrogen and stored at −80 °C. The brains were mounted in Tissue-Tek Optimal Cutting Temperature compound (Sakura Finetek, USA) and allowed to equilibrate in a cryostat at −18 °C for 30 min before being sliced as 40 μm coronal sections and floated in 48-well microplates filled with cryoprotectant (Watson et al., 1986). Slices were stored at −20 °C until further use. In order to generate reference maps, every fifth section was counterstained with 0.01% thionin for 3 min, serially dehydrated with ethanol (70%, 95%, 100%; 20 s each), cleared with X-3B solvent (BDH Chemicals, Australia) for 10 s and then coverslipped with Depex Mounting Medium (BDH Laboratory Supplies, Poole, UK).

Immunohistochemistry

Free-floating sections were removed from cryoprotectant and washed with 0.1 M PBS (3 × 5 min) then pre-blocked with 10% normal serum (Millipore, USA), 0.5% Triton X-100 (BDH Chemicals, Australia) and 0.1 M PBS for 30 min prior to being incubated overnight on a shaker at RT with chicken anti-GFP primary antibody (Abcam) in a 1:1000 dilution with 2% normal serum and 0.5% Triton X-100 in 0.1 M PBS. The following day, sections were washed in 0.1 M PBS (3 × 5 min) and incubated for 2 h on a shaker at RT with either (i) biotinylated goat anti-chicken IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) in a 1:500 dilution for DAB-based detection; or (ii) Alexa Fluor® 488-conjugated donkey anti-chicken secondary antibody (Invitrogen, CA, USA) at a 1:200 dilution for fluorescent visualisation. Following incubation with the secondary antibody, sections were washed in 0.1 M PBS, mounted on gelatin-subbed slides with 0.9% saline and then coverslipped with anti-fade mounting medium (Dako, North America Inc., CA, USA). Tissue was visualised using a Zeiss Axio Observer inverted fluorescence microscope.

For DAB-based detection, sections were incubated for 1 h on a shaker at RT with streptavidin horseradish peroxidase complex (ABC, Vector Laboratories, USA) in 0.1 M PBS after incubation with the biotinylated secondary. Sections were then washed in 0.1 M PBS (3 × 5 min) prior to being incubated with DAB (Sigma–Aldrich, St Louis, MO, USA) in 0.1 M PBS for 15 min on a shaker. Addition of 0.03% hydrogen peroxide initiated the brown chromogenic reaction which was terminated by washing with 0.1 M PBS. The sections were mounted on slides with 0.5% gelatin and allowed to air dry overnight. Once dry, the slides were serially dehydrated in ethanol (50%, 70%, 90%, 100%; 1 min each) and cleared in X-3B solvent (BDH Chemicals, Australia) for 3 min. Finally, slides were cover-slipped with Depex Mounting Medium (BDH Laboratory Supplies, Poole, UK). The slides were viewed under an Olympus BH-2 light microscope. Using the Mouse Brain Atlas (Paxinos and Franklin, 2004) as a guide, a comprehensive neuroanatomical map of OX_1R-expressing neurons throughout the brain was detailed based on GFP-IR observed in perikarya and fibres. In this regard, we used a semi-quantitative scale as follows: − <5 cells/section; + 5–30 cells/section; ++ 30–200 cells/section; +++ >200 cells/section.

Results

OX_1R distribution pattern in the mouse brain

Widespread GFP-IR was observed in numerous brain regions of the OX_1R-eGFP mice ($n = 7$ brains examined). Wildtype mice were also assessed as controls, in which no staining was observed ($n = 3$ brains examined). The distribution pattern of GFP-IR in both somata and fibres is summarised in Table 1, and compared to published data for expression of OX_1R mRNA and orexinergic innervation.

Olfactory system. In general, a low-to-moderate signal was observed throughout the olfactory cortex. The anterior olfactory nucleus and tenia tecta contained a moderate density of immunopositive cell bodies along with short dendritic processes. A low density of immunolabelled cell bodies and dendrites were observed in the most rostral aspects of the piriform cortex.