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DISTRIBUTION OF OREXIN-1 RECEPTOR-GREEN FLUORESCENT PROTEIN- (OX_1-GFP) EXPRESSING NEURONS IN THE MOUSE BRAIN STEM AND PONS: CO-LOCALIZATION WITH TYROSINE HYDROXYLASE AND NEURONAL NITRIC OXIDE SYNTHASE

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Abstract—We used a reporter mouse line in which green fluorescent protein (GFP) was inserted into the orexin-1 receptor (OX_1) locus to systematically map the neuroanatomical distribution of the OX_1 receptor in the mouse brainstem and pons. Here, we show that the OX_1 receptor is expressed in a select subset of medullary and pontine nuclei. In the medulla, we observed OX_1-GFP expression in the cuneate, gracile, dorsal motor nucleus of the vagus (10N), nucleus of the solitary tract and medullary raphe areas. In the pons, the greatest expression was found in the locus coeruleus (LC) and dorsal raphe nucleus (DRN). High to moderate expression was found in the pedunculopontine tegmental nucleus (PPTg), laterodorsal tegmental nucleus, A5 noradrenergic cell group (A5) and the periaqueductal gray. Double-labeling with neuronal nitric oxide synthase (NOS1) revealed extensive co-localization in cell bodies and fibers of the 10N, A5 cell group and the PPTg. Double-staining with tyrosine hydroxylase revealed extensive co-expression in the LC, DRN and the lateral paragigantocellularis cell group in the ventral medulla. Our findings faithfully recapitulate the findings of OX_1 mRNA expression previously reported. This is the first study to systematically map the neuroanatomical distribution of OX_1 receptors within the mouse hindbrain and suggest that this OX_1-GFP transgenic reporter mouse line might be a useful tool with which to study the neuroanatomy and physiology of OX_1 receptor-expressing cells. © 2014 Published by Elsevier Ltd. on behalf of IBRO.

Key words: orexin, receptor, immunohistochemistry, medulla oblongata, green fluorescent protein.

INTRODUCTION

The neuropeptides orexin-A and orexin-B are produced via the proteolytic cleavage of a 130 amino acid long prepro-orexin protein, coded by a single gene transcript into 33 and 28 amino acid peptides, respectively (Sakurai et al., 1998). The prepro-orexin gene and protein are expressed by subsets of neurons located around the fornix, lateral hypothalamic area and dorsomedial hypothalamus. While the distribution of these neurons is localized within the hypothalamus, they have widespread projections that extend throughout the central nervous system (Peyron et al., 1998; Date et al., 1999). Unsurprisingly, the orexinergic system has been implicated in a range of diverse functions including the control of the sleep–wake cycle (Inutsuka and Yamanaka, 2013; Xu et al., 2013), thermoregulation (Tupone et al., 2011), feeding (Kay et al., 2014), reward (Lawrence 2010; Xu et al., 2013), and neuroendocrine (Inutsuka and Yamanaka, 2013) and cardiovascular regulation (Antunes et al., 2001; Carrive, 2013; Ciriello et al., 2013).

The actions of both orexin-A and orexin-B are mediated by two different G-protein-coupled receptors: orexin-1 (OX_1) and orexin-2 (OX_2) (Sakurai et al., 1998). Pharmacological studies demonstrate that both orexin-A and orexin-B have similar affinities for OX_2 receptors, whereas orexin-A is 10-fold more selective for OX_1 receptors than orexin-B (Sakurai et al., 1998). As such, orexin-A is widely regarded as being selective for OX_1 receptors. Activation of OX_1 receptors within the ventrolateral periaqueductal gray has been shown to induce anti-nociception (Ho et al., 2011), while in the hindbrain, OX_1 receptors modulate meal size (Parise et al., 2001). In the rostral ventrolateral medulla (RVLM), OX_1 receptors have been suggested to be expressed on sympathoexcitatory bulbospinal neurons as exogenous application of orexin-A has been shown to elicit increases in blood pressure, sympathoexcitation and increases baroreflex sensitivity (Shahid et al., 2012).

There have been few studies that have mapped the distribution of the OX_1 and OX_2 receptor subtypes within the mammalian brain. There have, however, been comprehensive studies describing the distribution of the

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Abbreviations: 10N, dorsal motor nucleus of the vagus; A5, A5 noradrenergic cell group; Amb, nucleus ambiguus; AP, area postrema; BAC, bacterial artificial chromosome; DRN, dorsal raphe nucleus; GFP, green fluorescent protein; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; LPGi, lateral paragigantocellular nucleus; mRNA, messenger ribose nucleic acid; MVe, medial vestibular nucleus; NOS1, nitric oxide synthase, type 1; NTS, nucleus of the solitary tract; OX_1, orexin-1 receptor subtype; OX_2, orexin-2 receptor subtype; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PPTg, pedunculopontine tegmental nucleus; RVLM, rostral ventrolateral medulla; TH, tyrosine hydroxylase.

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OX_1 receptor messenger RNA (Marcus et al., 2001; Hervieu et al., 2001). This is most likely due to the fact that producing specific antibodies to G-protein-coupled receptors has been challenging. Many of the available antibodies appear to lack specificity and produce high levels of background staining. To circumvent this issue, we used an enhanced green fluorescent protein (GFP) reporter mouse to characterize the distribution of OX_1 receptors within the mouse brain stem and pons. Additionally, given the evidence for interactions between the orexinergic, nitrergic (Shih and Chuang, 2007; Xiao et al., 2013) and catecholaminergic (Shahid et al., 2012; Soya et al., 2013) systems, we also performed a systematic analysis of OX_1-GFP co-localization with the neuronal isoform of nitric oxide synthase (NOS1) and a synthetic enzyme for catecholamines, tyrosine hydroxylase (TH).

EXPERIMENTAL PROCEDURES

All experimental protocols used in this study were performed in accordance with the Prevention of Cruelty to Animals Act, Australia 1986, conformed with the guidelines set out by the National Health and Medical Research Council of Australia (2007), and were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee.

To determine the distribution of OX_1 receptors in the brainstem and pons, adult male transgenic OX_1-GFP (bacterial artificial chromosome (BAC)) reporter mice (founder line KP68Gsat/Mmed) from the Mutant Mouse Regional Resource Centers on a CD-1 background were used (n = 8). A GFP reporter gene, followed by a polyadenylation sequence, was inserted into BAC clone RP23–282L14 at the initiating ATG codon of the first coding exon of the Hcrtr1 gene. The modified BAC containing the inserted GFP upstream of the Hcrtr1 gene was injected into the pronuclei of FVB/N fertilized oocytes. Hemizygous progeny were mated to Crl:CD1(ICR) mice. The original founder animals were a gift from Dr. Danny Winder (Vanderbilt University, Nashville, TN, USA) and Dr. Paul Kenny (Mount Sinai School of Medicine, New York, NY, USA). All mice used in this study were males aged between 18 and 22 weeks. Mice were housed under 12-h–12-h light–dark conditions and allowed *ad libitum* access to standard laboratory chow (Barastoc, Australia) and tap water.

Perfusion

All perfusions were performed during the light phase (between 9:00 and 12:00). Mice (35–42 g) were deeply anesthetized with sodium pentobarbitone (100 mg/kg i.p.), and perfused with 50 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 90–120 mmHg of perfusion pressure followed by 50 ml of 4% paraformaldehyde (PFA; w/v; Merck Millipore). The brain was then removed and transferred to 4% PFA for 4 h then PBS containing 20% sucrose (w/v) and stored at 4 °C until processed.

Prior to sectioning, the brains were frozen over liquid nitrogen and four sets of coronal sections were cut (30-μm thickness) on a cryostat (Leica Cryocut 1850, Leica Microsystems, Wetzlar, Germany). The free-floating sections were collected in 24-well tissue culture plates containing PBS prior to being processed for immunohistochemical detection of GFP, NOS1 and TH.

Immunohistochemical detection of GFP

For immunohistochemical detection of GFP, we used a commercial chicken polyclonal GFP antisera directed against the full-length recombinant GFP protein (Abcam, Cambridge, UK; catalog number: ab13970, lot number: 13879-5). The specificity of this antibody has been extensively characterized in the past and has been shown to be highly specific (Llewellyn-Smith et al., 2011; Leinders-Zufall et al., 2014). Immunohistochemistry was performed as described previously (Yao et al., 2012). Free-floating mouse brain stem sections were incubated for 10 min in a blocking solution comprising 10% normal goat serum (NGS, Sigma–Aldrich, MO, USA) and 0.3% Triton X-100 (Sigma–Aldrich) in 0.1 M PBS followed by rinses in PBS (3 × 10 min). Sections were then incubated in a polyclonal chicken anti-GFP primary antiserum (1:10,000 dilution) in PBS containing 0.3% Triton X-100 for 24 h at 4 °C. After the primary antibody incubation, sections were rinsed in PBS (3 × 10 min) prior to a 1-h incubation in PBS containing biotinylated goat anti-chicken IgG (1:500 dilution, Jackson Laboratories, PA, USA), in PBS/0.3% Triton X-100 at room temperature. Following rinses in PBS (3 × 10 min), the sections were incubated for 1 h in streptavidin-conjugated Alexfluor-488 (1:500 dilution, Life Technologies, UK) in PBS containing 0.3% Triton X-100. Subsequent to further washes (3 × 10 min) the sections were mounted onto microscope slides with 0.5% gelatin solution and coverslipped using anti-fade mountant containing 4′,6-diamidino-2-phenylindole (DAPI; Fluoroshield ™, Sigma–Aldrich, Clayton, Australia).

GFP, NOS1 and TH triple fluorescence immunohistochemistry

In order to determine whether OX_1-GFP-expressing neurons also express NOS1 and/or TH, we performed triple-labeling fluorescence immunohistochemistry. The NOS1 and TH staining was carried out using a mouse anti-NOS1 antibody (Santa Cruz Biotechnology, CA; catalog number: sc-5302, lot number: E2610) and a rabbit anti-TH antibody (Merck Millipore, Kilsyth, Australia; catalog number: AB152, Lot number: 2219275), both extensively characterized in the past (Wang et al., 2011; Szot et al., 2012). To confirm the presence of OX1-GFP expression in neurons we used a mouse anti-nuclear antigen N (NeuN; 1:1000 dilution; Merck Millipore, Kilsyth, Australia, catalog number: MAB377). A rabbit anti-glial fibrillary acidic protein (GFAP; 1:2000 dilution; Dako, Sydney, Australia, catalog number: Z0334) was also used to determine whether OX1-GFP was expressed in glia.

After sectioning, brainstem slices were rinsed with PBS containing Triton X-100 (0.3%) for 10 min. Sections were then incubated with the primary antisera (Chicken

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