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The generation of knock-in mice expressing fluorescently tagged galanin receptors 1 and 2

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ABSTRACT

The neuropeptide galanin has diverse roles in the central and peripheral nervous systems, by activating the G protein-coupled receptors Gal_1, Gal_2 and the less studied Gal_3 (*GalR1–3* gene products). There is a wealth of data on expression of Gal_{1-3} at the mRNA level, but not at the protein level due to the lack of specificity of currently available antibodies. Here we report the generation of knock-in mice expressing Gal_1 or Gal_2 receptor fluorescently tagged at the C-terminus with, respectively, mCherry or hrGFP (humanized *Renilla* green fluorescent protein). In dorsal root ganglia (DRG) neurons expressing the highest levels of Gal_1-mCherry, localization to the somatic cell membrane was detected by live-cell fluorescence and immunohistochemistry, and that fluorescence decreased upon addition of galanin. In spinal cord, abundant Gal_1-mCherry immunoreactive processes were detected in the superficial layers of the dorsal horn, and highly expressing intrinsic neurons of the lamina III/IV border showed both somatic cell membrane localization and outward transport of receptor from the cell body, detected as puncta within cell processes. In brain, high levels of Gal_1-mCherry immunofluorescence were detected within thalamus, hypothalamus and amygdala, with a high density of nerve endings in the external zone of the median eminence, and regions with lesser immunoreactivity included the dorsal raphe nucleus. Gal_2-hrGFP mRNA was detected in DRG, but live-cell fluorescence was at the limits of detection, drawing attention to both the much lower mRNA expression than to Gal_1 in mice and the previously unrecognized potential for translational control by upstream open reading frames (uORFs).

1. Introduction

Galanin is a 29–30 amino acid neuropeptide that is dramatically induced after peripheral or central nervous system injury, and plays physiological roles in nociception, memory and cognition, anxiety-related behaviours, feeding, reproduction, neurite outgrowth and as a neuronal trophic factor (Cortes et al., 1990; Lang et al., 2015; Villar et al., 1989; Webling et al., 2012). The N-terminal 15 residues of galanin are strictly conserved between species and the N-terminal end is essential for biological activity (Lang et al., 2015; Webling et al., 2012), binding to the three galanin receptor subtypes Gal_1, Gal_2 and Gal_3 (*GalR1–3* gene products), which are each Class A rhodopsin-like G protein-coupled receptors (GPCRs) that differ in sites of expression, functional coupling and signalling activities (Webling et al., 2012). The phenotypes of mice deficient in each of the galanin receptors have recently been reviewed (Brunner et al., 2014; Lang et al., 2015; Webling et al., 2012). Gal_{1-3} are also bound by galanin-like peptide (GALP), but not by the GALP alternatively spliced product alarin (Webling et al., 2012), and recently the neuropeptide spexin/NPQ (neuropeptide Q) was also reported to bind to Gal_2 and Gal_3 , but not to Gal_1 (Kim et al., 2014).

In adult rat the expression of Gal_1 mRNA is largely restricted to brain, spinal cord and dorsal root ganglia (DRG), whereas Gal_2 mRNA is also detected in several peripheral tissues such as large intestine and uterus. In contrast, Gal_3 mRNA has a more restricted distribution within brain,

Abbreviations: AOTF, acousto-optic tunable filter; BAC, bacterial artificial chromosome; CDS, coding sequence; CMV, cytomegalovirus; DOR, δ-opioid receptor; DR, dorsal raphe nucleus; DRG, dorsal root ganglia; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; ES cell, embryonic stem cell; FRT sites, FLP recombination target sites; GALP, galanin-like peptide; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GPCRs, G protein-coupled receptors; hrGFP, humanized *Renilla* green fluorescent protein; ISH, in situ hybridization; LSN, lateral spinal nucleus; ME, median eminence; NPY, neuropeptide Y; nt, nucleotides; RNA-seq, next generation RNA sequencing; RT-PCR, reverse transcription polymerase chain reaction; TSA, tyramide signal amplification; UTR, untranslated region; uORFs, upstream open reading frames.

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is rare in spinal cord and rare or not present in DRG, and expression in peripheral tissues is controversial (Burazin et al., 2000; Howard et al., 1997; O'Donnell et al., 1999; Shi et al., 2006; Waters and Krause, 2000; Webling et al., 2012). By in situ hybridization (ISH), Gal_1 mRNA is more highly expressed than Gal_2 overall in brain (Burazin et al., 2000) and by far the highest levels of Gal_2 detected in the nervous system are in the DRG (O'Donnell et al., 1999).

In adult mouse brain the distribution of Gal_1 mRNA by ISH is largely similar to rat (Hohmann et al., 2003), whereas the absence of specific [^{125}I]-galanin binding sites in any region of adult Gal_1-deficient brain (Jungnickel and Gundlach, 2005) suggests a species-specific, greatly reduced expression of Gal_2 mRNA. However, it is still detectable by the more sensitive RT-PCR method in both mouse whole brain (Hobson et al., 2006; Jacoby et al., 2002) and subregions including the amygdala, hippocampus and hypothalamus (Brunner et al., 2014; Hawes et al., 2005; He et al., 2005; Shi et al., 2006; Zhao et al., 2013), as well as in spinal cord (Jacoby et al., 2002), DRG, trigeminal and nodose sensory ganglia (Hobson et al., 2006; Page et al., 2007) and several peripheral tissues (Barreto et al., 2011; Hobson et al., 2006; Jacoby et al., 2002; Kim and Park, 2010; Pang et al., 1998). Less work has been reported on Gal_3 mRNA expression, but by RT-PCR it is detected in mouse whole brain and some subregions (Brunner et al., 2014; Hawes et al., 2005; Zhao et al., 2013), nodose ganglion (Page et al., 2007) and several peripheral tissues (Barreto et al., 2011; Brunner et al., 2014; Kim and Park, 2010), but is at the limits of detection in both spinal cord and DRG (Hobson et al., 2006; Jacoby et al., 2002).

Current antibodies against Gal_1 or Gal_2 are non-selective under standard immunodetection conditions, with identical immunoreactivity patterns in wild-type and receptor knockout mice (Hawes and Picciotto, 2005; Lang et al., 2015; Lu and Bartfai, 2009; F.E.H., P.V. and D.W., unpublished). To delineate the expression of Gal_1 and Gal_2 at the protein level we wished to tag each receptor with fluorochromes. The C-terminal tagging of GPCRs with green fluorescent protein (GFP) is generally thought to have no significant effect on GPCR properties e.g. ligand binding, signal transduction and intracellular trafficking (Ceredig and Massotte, 2014), and both Gal_1 and Gal_2 have been shown to be functional when C-terminally tagged with enhanced GFP (EGFP) or its variants and expressed in cell lines (Wirz et al., 2005; Xia et al., 2004, 2008). The GPCR superfamily is the largest group of cell surface receptors and are the targets of around one third of marketed drugs, yet to date the only knock-in mice that express a fluorescently-tagged GPCR are Rhodopsin-EGFP and two rhodopsin mutant variants, δ-opioid receptor (DOR)-EGFP and the recently reported μ-opioid receptor (MOR)-mCherry (Ceredig and Massotte, 2014; Erbs et al., 2015; Scherrer et al., 2006). Transgenic mice have been successfully generated expressing either humanized *Renilla* GFP (hrGFP; Stratagene-Agilent; Zeng et al., 2003) under the control of various endogenous promoters (Sakata et al., 2009; van den Pol et al., 2009; Voigt et al., 2012), or with widespread expression of the monomeric red fluorescent protein mCherry under the control of a ubiquitin-C promoter (Fink et al., 2010; Shaner et al., 2004). Here we describe the generation and initial characterization of Gal_1-mCherry and Gal_2-hrGFP knock-in mice, focussing on expression in DRG, spinal cord and brain.

2. Materials and methods

2.1. DNA sequence analysis

Planning for knock-in gene characterizations used mouse reference genome Build 37.1, vector hrGFP-FRT*neo*FRT (see below, Section 2.2) and mCherry cDNA (AY678264) sequences. The RepeatMasker 3.2.9 programme (Smit, Hubley and Green, 1996–2010, http://www.repeatmasker.org) was used to select regions of the *GalR1* or *GalR2* genes for retrieval target sites or Southern probes that avoided repetitive DNA elements, and homology arm targets that minimized the presence of repetitive DNA elements. Ribosome profiling data (Ingolia et al., 2011) was accessed using the GWIPS-viz. browser (http://gwips.ucc.ie; Michel et al., 2014). The ribosome density peaks for the uORF6 and *GalR2* initiation codons were at, respectively, Chr11 nucleotides (nt) 116,281,254–286 and 116,281,474–509 of the GRCm38/mm10 genome assembly (GWIPS-viz. ribo-seq coverage plot; Ingolia et al., 2011; Michel et al., 2014).

2.2. Generation of GalR1-mCherry-[neo$^+$] and GalR2-hrGFP-[neo$^+$] knock-in mice

Mouse genomic clones including either *GalR1* or *GalR2* genes from the bMQ mouse strain 129S7 (129Sv) bacterial artificial chromosome (BAC) library (inserts 89–178 kb; Source BioScience; Adams et al., 2005) were electroporated into strain EL250 *Escherichia coli* (Lee et al., 2001). This allowed temperature-inducible, lambda Red-mediated, homologous recombination into the BAC (Copeland et al., 2001; Lee et al., 2001) of PCR products from either vector hrGFP-FRT*neo*FRT (Balthasar et al., 2004; Parton et al., 2007; hrGFP derived from Stratagene-Agilent vector phrGFP-1, Zeng et al., 2003; Fig. 2A, *middle panel*) flanked by *GalR2* homologous sequence or vector mCherry-FRT*neo*FRT (hrGFP exchanged for mCherry; Shaner et al., 2004) flanked by *GalR1* homologous sequence. Within the latter PCR product an AseI restriction site was introduced immediately downstream of the 3′ FRT site (Fig. 1A, *middle panel*), for use in Southern blot digests, and the mCherry/hrGFP heterologous 3′-untranslated region (UTR) has identity to nt 705–1193 of vector pCMV-Script (AF028239) which includes the SV40 early region poly(A) site (Connelly and Manley, 1988; J02400, nt 2828–2547). Correct insertion into the BAC was validated by DNA sequencing of cloned PCR products of each junction. All DNA sequencing was by Source BioScience, Oxford.

Retrieval from the recombined BAC clones by gap repair (Copeland et al., 2001; Lee et al., 2001) into PCR-amplified vector pCR-Blunt (Invitrogen) was mediated by either *GalR1* target sequences with adjacent introduced rare SwaI restriction sites, or *GalR2* target sequences with adjacent introduced BstZ17I restriction sites. Recombined plasmid DNAs were transformed into STBL3 *E. coli* (Invitrogen) and the final targeting constructs were excised with either SwaI (*GalR1*-mCherry-FRT*neo*FRT; 11.3 kb, with *GalR1* homology arms of 4.2 and 3.9 kb) or BstZ17I (*GalR2*-hrGFP-FRT*neo*FRT; 8.1 kb, with *GalR2* homology arms of 2.3 and 2.7 kb).

Targeting construct inserts were electroporated into embryonic stem (ES) cell line E14.1a (strain 129P2/OlaHsd; Downing and Battey, 2004; Hooper et al., 1987) and the SV40-*neo* cassette selected with 250 μg/ml G418 by Geneta (Dept. of Biochemistry, University of Leicester). G418-resistant ES cell clones were screened for correct targeting by PCR (data not shown) and real-time quantitative genomic PCR, and expanded clones were screened by Southern blot analysis (Supplementary Materials and Methods). Selected clones were karyotyped to confirm euploidy, and three ES cell clones of each knock-in gene were injected into 3.5 day old blastocysts from C57BL/6J mice to produce chimeric mice (Geneta). These were crossed to strain 129P2/OlaHsd mice and germline transmission was assessed by PCR genotyping (see below).

2.3. Animals

Mice were housed in a temperature- and humidity-controlled colony on a 14:10 h light–dark cycle, and fed standard chow and water ad libitum. Procedures were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. Ear-punch biopsies were used for PCR genotyping, animals were killed by cervical dislocation to obtain DRG for RT-PCR analysis (Section 2.6), or primary DRG cultures (Section 2.7). Three mice had peripheral transection of the right sciatic nerve prior to perfusion seven days later to obtain ipsilateral (axotomized) lumbar L4 and L5 DRG (Holmes et al., 2008) for immunohistochemistry (Section 2.9).