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SHORT COMMUNICATION

# Analyzing the validity of GalR1 and GalR2 antibodies using knockout mice

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**Abstract** G-protein-coupled receptors (GPCRs) comprise the largest family of cell surface receptors and are the major drug targets for the treatment of various human diseases. The lack of sensitive and selective antibodies capable of recognizing endogenous GPCRs, however, hampers the progress of research on this class of receptors. GalR1 through GalR3, GPCRs for the neuropeptide galanin, are potential drug targets for seizure, Alzheimer's disease, depression and anxiety, as well as pain and metabolic syndrome; therefore, determining the cellular and subcellular localization of galanin receptors is of high interest. Several Antibodies raised against galanin receptors are currently available from commercial or academic sources. We have tested several antibodies to GalR1 and GalR2 on tissues from respective knockout mice. Unexpectedly, the immunoreactivity patterns are the same in wild-type and in knockout mice, suggesting that current GalR1 and GalR2 antibodies, under standard immunodetection conditions, might not be suitable for mapping the receptors. These findings argue for taking precaution when using antibodies to galanin receptors.

**Keywords** Galanin · Antibody · CNS

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## Introduction

GPCRs are integral membrane proteins with similar structures involving seven transmembrane domains, three intracellular loops, three extracellular loops, an extracellular N-terminal domain, and an intracellular C-terminal domain. The immunohistochemical mapping of the receptor distribution requires generation of specific antibodies to the GPCRs. The most common strategy to generate antibodies to GPCRs has been to synthesize peptide antigens of 10 to 40 amino acids, typically fragments of the N-terminal domain, the second or third intracellular loop, or the C-terminal domain of the GPCRs. This strategy has been used in immunohistochemical mapping of close to 200 GPCRs.

The traditional method to confirm the specificity of an antiserum is to pre-absorb the antiserum with the synthetic peptide and check for the disappearance of the immunoreactivity in the Western blot and immunohistochemistry. Numerous articles have been published in the literature using antibodies that were validated with antigen peptide pre-absorption. Some of these articles, on the contrary to the intention of the authors, might have provided us with erroneous information regarding the distribution of the GPCR of interest. This is because the small peptide antigens, which only contain a small fraction (ca 5–10%) of the whole peptide sequence, might not be able to replicate the secondary and tertiary structures unique for the GPCR antigen of interest. Indeed, the safety of peptide pre-absorption as a sole confirmation of antibody specificity is in jeopardy with the generation of numerous GPCR receptor knockout (KO) mice, which, both intuitively and practically, represent ideal tools for testing the specificity of GPCR antibodies.

The neuropeptide galanin is involved in the regulation of several CNS and PNS processes such as cognition, seizure control, feeding, mood regulation, addiction and reward, as well as pain transmission (reviewed by Leibowitz 1989; Bartfai et al. 1993; Hokfelt et al. 1998; Lu et al. 2007; Counts et al. 2008; Crawley 2008; Kuteeva et al. 2008; Lerner et al. 2008; Picciotto 2008). Based on these functional findings, determining the distribution of the three known galanin receptors, GalR1–GalR3, is of high interest. Over the years, much effort has been devoted to generate antibodies specific for the galanin receptors and map their distribution in the rodent brain aiming to better understand the role of the galanin system in CNS physiology and pathology. The GalR1KO and different strains of GalR2KO mice became available in 2002 (Jacoby et al. 2002) and 2005 (Gottsch et al. 2005; Shi et al. 2006; Bailey et al. 2007; Elliott-Hunt et al. 2007; Lu et al. 2008), respectively. These mice strains provide us with excellent tools for analyzing the validity of the currently available GalR1 and GalR2 antibodies.

## Materials and methods

### Western blot

Tissues from mice hypothalamus were homogenized on ice in 0.32 M sucrose containing 50 mM Tris–HCl, 5 mM EDTA, and 1 tablet of mixture protease inhibitors/50 ml (Roche Applied Science), pH 7.4, using a 2-ml homogenizer, centrifuged at 1,000×*g* for 10 min at 4°C to remove the nuclear fraction. The supernatant was recentrifuged at 50,000×*g* for 20 min and the resultant pellet was resuspended in 40 mM Tris–HCl, pH 7.4, by sonication. After determination of protein concentration, the appropriate amount of 5× Laemmli sample buffer was added and the samples were heated for 10 min at 70°C and subjected to SDS-PAGE and Western blotting. The blocking solution and antibody dilution solution were both 5% bovine serum albumin/Tris-buffered saline (pH 7.4). The ADI-R1, Ab96125, AB9883 were used at a dilution of 1:500, 1:5,000, and 1:3,000, respectively, at 4°C overnight. Chemiluminescent detection was performed using the SuperSignal Western Pico reagent (Pierce, Rockford, IL). The experiments were repeated at least three times.

### Immunohistochemistry

Adult male mice were deeply anesthetized and perfused with 4% paraformaldehyde, and 30-μm coronal sections were cut with a cryostat. Briefly, free-floating sections were first incubated in 3% normal goat serum diluted in PBS with 0.1% Triton X-100 for 1 h at room temperature, followed by incubation with antibodies against GalR1 (ADI-R1, 1:1000) or GalR2 (Ab9883, 1:1,000) overnight at 4°C, followed by secondary antibodies conjugated to FITC (Jackson ImmunoResearch) for 30 min at 37°C. The sections were examined with a confocal scanning microscope (Olympus, Melville, NY) equipped with the appropriate filter combinations. Six wild-type and six knockout mice were used for immunodetection and the representative results were shown in Fig. 1a.

## Results

We have tested anti-GalR1 (ADI-R1, catalog # GALR11-A) and anti-GalR2 (catalog # GALR21-A) antibodies from Alpha Diagnostic International (San Antonio, TX); anti-GalR1 (catalog # AB5646P, to N-terminal immunogen), anti-GalR2 (catalog # AB5648P, to C-terminal immunogen) antibodies from Chemicon (Temecula, CA), and anti-GalR1 (Ab96125, to C-terminal immunogen) and anti-GalR2 (Ab9883) from CURE at UCLA (Los Angeles, CA), using GalR1 KO and GalR2 KO mice. Some of the representative results are summarized in Fig. 1. All antibodies used in these studies were polyclonal antibodies raised against immunogens 10 to 22 amino acids in length corresponding to mouse galanin receptor sequences.

In Western blot, the GalR1 antibodies ADI-R1 and Ab96125 recognized a band in hypothalamic lysate from wild-type mice that migrated at ~40 kDa. Unexpectedly, this band was also present in the GalR1KO hypothalamic lysate. Similarly, the protein species in the wild-type hypothalamic lysate recognized by GalR2 Ab9883, a band around 60 kDa and a band around 40 kDa, were present in the GalR2KO hypothalamic lysate.

Immunohistochemistry performed on the hippocampal region of the mouse brain with ADI-R1 predominantly labeled the CA3 region; however, the pattern of the wild-type and knockout sections was identical. Finally, the GalR2 antibody Ab9883 stained the dentate gyrus and CA region of the wild-type and knockout mice in a similar pattern as well.

## Discussion

We have tested several GalR1 and GalR2 antibodies that are available from commercial or academic sources using GalR1KO and GalR2KO mice. We have chosen to use hypothalamic and hippocampal tissues because both tissues are known to express GalR1 and GalR2 receptors at significant levels (Hohmann et al. 2003; He et al. 2005; Shi et al. 2006). In both Western blot and immunohistochemistry, the immunoreactivity patterns of GalR1or GalR2

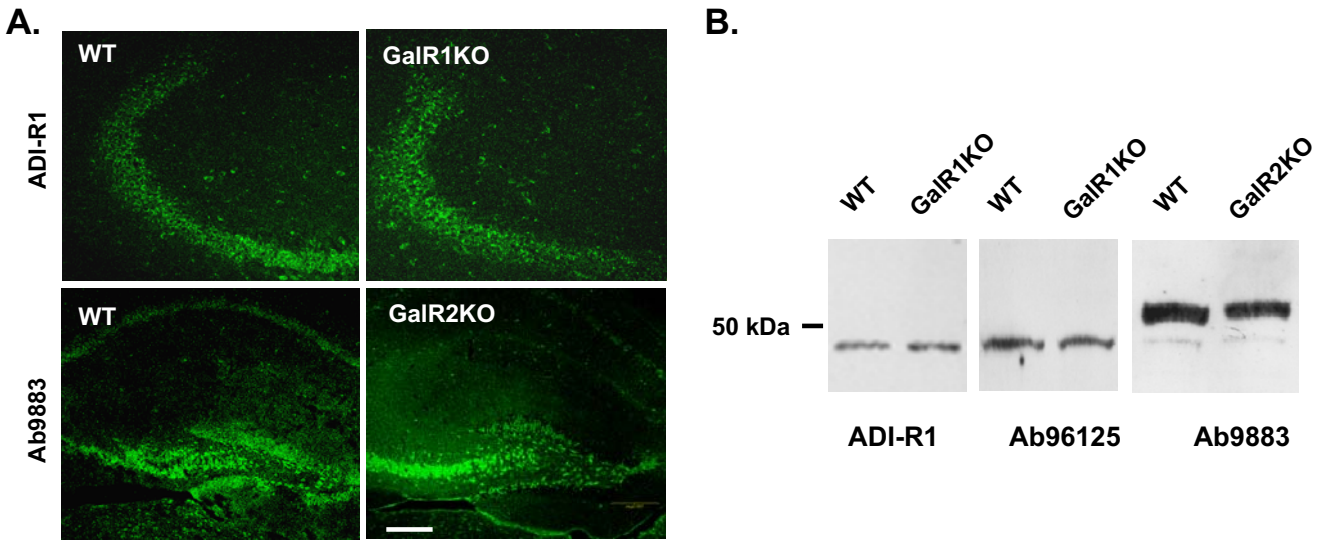


**Fig. 1** Analyzing the validity of GalR1 and GalR2 antibodies using knockout mice. **a** Sections containing dorsal hippocampus from wild-type, GalR1 (−/−) or GalR2 (−/−) mice were stained with antibodies against GalR1 (ADI-R1, 1:1,000) or GalR2 receptors (Ab9883 1:1,000). *Scale bar* 100 μm. **b** Hypothalamic lysates (15 μg) from wild-type, GalR1 (−/−) and GalR2 (−/−) mice were processed for Western blot analysis. Primary antibodies against GalR1 (ADI-R1, 1:500; Ab96125, 1:5,000) and GalR2 (Ab9883, 1:3,000) were used for immunodetection

antibodies were identical in wild-type and knockout mice. These results were similar to those reported by Hawes and Picciotto (2005) in which a total of three antibodies, ADI-R1 (GalR1), Ab 9883 (GalR2) and, K-20 (GalR2; Santa Cruz, CA), all exhibit similar immunoreactivity patterns in wild-type and knockout mice in the Western blot.

The GalR1KO mouse was generated by exon 1 disruption at the KpnI site that is ~600 bp downstream from the start codon (Jacoby et al. 2002). The GalR2 knockout mouse strain used in our study was generated by insertion of a targeting vector in the first intron of GalR2 gene and the absence of full length GalR2 transcript was confirmed by RT-PCR using primers that correspond to sequences on the two different exons (Shi et al. 2006). The epitope of Ab96125 corresponds to C-terminal region of GalR1 (Larm et al. 2003), which is downstream from the disruption site in the GalR1 knockout mice; therefore, specific antibody binding is not expected. The precise epitope locations of commercial antibodies were not disclosed; however, since the molecular weight of labeled bands in Western blots were matched between wild-type and knockout mice, the labeled bands seen in the knockout mice are unlikely to be the truncated or aberrant form of galanin receptors. Rather, the detected species are most likely proteins structurally related to the epitope. Among many possibilities, the reason the protein of interest, in this case GalR1 or GalR2, was not labeled could be, under the experimental conditions, the endogenous GalR1 and GalR2 assumed a complex configuration distinctive from the immunogens to which the antibodies were raised. Alternatively, the binding of antibodies to the nonspecific protein species prevented them from binding to GalR1 or GalR2. The argument against the latter possibility is that for each antibody assessed, we have tested at least three different concentrations (data not shown), typically ranging from 1:100 to 1:5,000. In each condition tested, we were unable to detect any band/protein that is present in wild-type but absent in knockout mice.

As we have only tested the antibodies on the paraformaldehyde fixed free-floating sections in immunohistochemistry and with a standard protocol in Western blot, it remains open that certain protocols might be able to label galanin receptors with these antibodies in a sensitive and specific manner. Nevertheless, we speculate that even if such an ideal working condition is achieved for a specific "difficult" antibody, it might not be able to provide a comfortable window to allow reliable and consistent labeling when small fluctuations in experimental conditions commonly occur from batch to batch. Therefore, it is still desirable to run a knockout mouse control for "difficult" antibodies whenever possible.

Our data, together with those previously reported (Hawes and Picciotto 2005), question the validity of using existing GalR antibodies for standard immunodetection. The powerful way neuroanatomy guides our thinking about the functions of GPCRs in the nervous system makes an accurate localization of receptors essentially important. Without a reliable antibody, this information will have to be deduced from a congruence of information, such as in situ hybridization, Northern blot, isotope-labeled ligand binding, as well as functional studies. Nevertheless, antibodies remain the best tool for precise localization of the

protein of interest. Therefore, continuous efforts are needed to develop sensitive and specific antibodies to galanin receptors and we recommend that the all newly developed antibodies be validated with knockout tissues (if available) before their application.

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