SUBUNIT-SPECIFIC POLYCLONAL ANTIBODY TARGETING HUMAN ρ1 GABA_C RECEPTOR

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Abstract

The GABA_C receptor, a postsynaptic membrane receptor expressed prominently in the retina, is a ligand-gated ion channel that consists of a combination of ρ subunits. We report characterization of a novel guinea pig polyclonal antibody, termed GABA_C Ab *N*-14, directed against a 14-mer peptide (*N*-14) of the extracellular domain of the human ρ1 subunit. The antibody exhibits high sensitivity for *N*-14 by ELISA. In Western blots, GABA_C Ab *N*-14 shows reactivity with the ρ1 subunit of preparations obtained from ρ1 GABA_C-expressing neuroblastoma cells, *Xenopus* oocytes, and mammalian retina and brain. Flow cytometry reveals a rightward shift in mean fluorescence intensity of GABA_C-expressing neuroblastoma cells probed with GABA_C Ab *N*-14. Immunostaining of neuroblastoma cells and oocytes with GABA_C Ab *N*-14 yields fluorescence only with GABA_C-expressing cells. Antibody binding has no effect on GABA-elicited membrane current responses. Immunostaining of human retinal sections shows preferential staining within the inner plexiform layer. GABA_C Ab *N*-14 appears well suited for investigative studies of GABA_C ρ1 subunit in retina and other neural tissues.

Keywords

GABA receptor; GABA_C; retina; ρ1 subunit; antibody

INTRODUCTION

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter of the central nervous system. Postsynaptic receptors of the GABA_A family are pentameric ligand-gated chloride channels. GABA_C receptors, a sub-family of GABA_A, consist of ρ subunits and are expressed in brain tissue, with especially high expression in retina (Amin and Weiss, 1996;

Boue-Grabot et al., 1998; Cutting et al., 1991; Drew, and Johnston, 1992; Enz et al., 1996; Euler and Wässle, 1998; Feigenspan et al., 1993; Lukasiewicz and Shields, 1998; Polenzani et al., 1991; Qian and Dowling, 1993; Schlicker et al., 2009; Zhang et al., 1995). Three types of GABA ρ subunits (ρ1, ρ2, and ρ3) have been cloned from mammalian retina, with the ρ1 subunit exhibiting much higher expression level than ρ2. It is well documented that ρ1 subunits can assemble to form functional homopentameric receptors (Cutting et al., 1991; Qian et al., 1998; Zhang et al., 1995).

The availability of an antibody to the human GABA$_C$ ρ1 subunit would facilitate research on ρ1-containing GABA$_C$ receptors, and potentially also clinical studies aimed at selectively targeting tissues rich in ρ1. To serve as an anchor to target GABA$_C$-expressing cells, this antibody should be directed against an extracellularly accessible epitope, and antibody binding should preserve receptor function. Here we describe a novel polyclonal antibody directed against the extracellular domain of human ρ1 GABA$_C$, properties of which encourage its further use in immunolabeling and physiological studies. Preliminary accounts of this work have been reported (Gussin et al., 2008; Pending U.S. Patent Application number US2009/0269786 A1).

MATERIAL AND METHODS

A 14-mer peptide (*N*-14) consisting of the amino acid sequence RQRREVHEDAHKQV, located within the *N*-terminal region of the human ρ1 subunit, was chosen as the target for the antibody based on several considerations. First, this sequence lies outside the "core peptide" region, i.e., is not part of the more conserved region believed to be involved in inter-subunit interaction, ligand binding, and channel formation. Second, the 14-mer sequence resides within the "unstructured tail" of the *N*-terminal region, which is less conserved among species; BLAST/Blastp analysis of this region did not detect a putative conserved domain. Third, a search for the *N*-14 sequence using the NCBI website (blast.ncbi.nlm.nih.gov) yielded matches to ρ1 GABA$_C$ sequences for human and other species, and to *Burkholderia phymatum* hydrolase (9/14), but not to sequences for ρ2 GABA$_C$ or other GABA receptor subunits or to any other proteins. Thus, an antibody raised against this selected sequence conceivably could exhibit little cross-reactivity with other proteins.

Guinea pig was chosen as the species for antibody production. Peptide synthesis, conjugation to keyhole limpet hemocyanin (KLH), animal immunization and serum collection were contracted to an outside source (Covance, Inc., Denver, PA). IgG was purified from guinea pig serum by affinity chromatography (Borg et al., 1993), using protein A-Sepharose CL-4B beads (Sigma, St. Louis, MO). Peptide-specific antibody was further purified by affinity column chromatography (Affi-Gel 10 beads; Bio-Rad Laboratories, Hercules, CA); column-bound *N*-14 peptide served as ligand (Khasawneh et al., 2006). Following elution with glycine (100 mM, pH 2.5) and neutralization with 1 M Tris-base (pH 8.0), eluted fractions were analyzed for absorbance (280 nm); fractions of highest absorbance were pooled and dialyzed in PBS. The final concentration of the affinity-purified antibody, henceforth termed GABA$_C$ Ab *N*-14, was 0.24 mg/mL.

ELISAs were performed using microtiter wells pre-coated with neutravidin (Pierce – Thermo Fisher Scientific, Rockford, IL). Wells were either untreated (i.e., neutravidin only), or treated with either *N*-14 biotinylated peptide (100 µL, 10 µg/mL) or an unrelated biotinylated peptide (RSHSYPAKK). GABA$_C$ Ab *N*-14 was tested at 1/5,000 to 1/150,000 dilution. Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG (Santa Cruz Biotechnologies, Santa Cruz, CA), 1/2,000.

Neuroblastoma cells stably transfected to express human ρ1 GABA$_C$ (SHp5-human ρ1) were a gift from Dr. David S. Weiss (University of Texas Health Science Center at San Antonio). Non-expressing neuroblastoma cells (SHSY5Y; ATCC, Manassas, VA) were used as controls. *Xenopus laevis* oocytes expressing human ρ1 GABA$_C$, human ρ2, or α1β2γ2 GABA$_A$ receptors (α1, β2 and γ2 subunit sequences: rat, rat and human. respectively) were prepared as described (Gussin et al., 2006; Qian et al., 1997; Vu et al., 2005). Membrane protein preparations of *Xenopus* oocytes were obtained using previously described procedures (Wible et al., 1998).

Human retina was obtained from donor eye tissue (Illinois Eye-Bank, Bloomington, IL), in accordance with institutional policies. All procedures involving experimental animals conformed to institutional policies and to the Statement for the Use of Animals in Ophthalmic and Vision Research adopted by the Association for Research in Vision and Ophthalmology. Retina and brain tissues were lysed in RIPA buffer (Sefton, 2005). Human retinas were fixed in 4% paraformaldehyde, cryopreserved in sucrose, then sliced in Optimal Cutting Temperature medium (OCT, Tissue-Tek). The thickness of the retinal cryosections was 16 µm.

Western blots (15–25 µg protein per lane) were probed with GABA$_C$ Ab *N*-14 (1/10,000 dilution) and, as secondary antibody, HRP-conjugated goat anti-guinea pig IgG (1/7,000 dilution; Santa Cruz Biotechnology). Controls involved probing with secondary antibody only, or with GABA$_C$ Ab *N*-14 that had been pre-absorbed with *N*-14 (3 µg/mL, 30 min, room temperature), followed by secondary antibody. Control rabbit anti-human GABA$_C$ ρ2 polyclonal antibody was obtained from Abcam (Cambridge, MA).

Flow cytometry was performed on SHp5-ρ1 neuroblastoma cells that express ρ1 GABA$_C$, and on control SHSY5Y cells. Cells were incubated with GABA$_C$ Ab *N*-14 (1/25 to 1/1,000 dilution) or (as control) normal guinea pig IgG (1/100) as primary antibody, and with FITC-conjugated goat anti-guinea pig IgG (Santa Cruz Biotechnology; 1/50 dilution) as secondary antibody. Single-color analysis employed a FACStar flow cytometer (BD Biosciences, San Jose, CA). A lower-limit threshold was set for data acquisition, thereby eliminating background scatter.

Immunofluorescence labeling of live SHp5-ρ1 and SHSY5Y cells employed GABA$_C$ Ab *N*-14 (1 hr, 1/1,000 – 1/2,000 dilution) as primary antibody and biotinylated goat anti-guinea pig IgG secondary antibody (Santa Cruz Biotechnology; 45 min, 1/400 dilution), followed by 10 nM streptavidin-conjugated quantum dots 605 (SA-qdots; Invitrogen, Carlsbad, CA; 15 min). Oocytes expressing either GABA$_C$ ρ1 or α1β2γ2 GABA$_A$ receptors, non-expressing control oocytes, and human retinal sections were incubated (1–2 h) with either GABA$_C$ Ab *N*-14 (1/1,000), with GABA$_C$ Ab *N*-14 that had been pre-absorbed with 0.1 mg/mL of *N*-14 peptide (45 min, room temperature), or without GABA$_C$ Ab *N*-14. The secondary antibody was goat anti-guinea-pig IgG (Abcam) (FITC-conjugated, 1/200 for human retina sections; Cy5-conjugated, 1/400 for oocytes; 1-h incubation). Slides were mounted using Vectashield H-1000 medium (Vector Laboratories, Burlingame, CA), and images obtained on a Leica DM-IRE2 confocal microscope at 20X (oocytes) or 40X (retinal sections) magnification.

GABA-elicited membrane current responses were recorded from GABA$_C$ ρ1 expressing oocytes and from SHp5-ρ1 cells using, respectively, two-electrode voltage clamp and whole cell patch-clamp configurations (Wotring et al., 2003; Vu et al., 2005). Prior to electrophysiological testing, cells were subjected to conditions similar to those used for immunofluorescence (untreated; incubated with GABA$_C$ Ab *N*-14 only; or incubated with GABA$_C$ Ab *N*-14 followed by secondary antibody).

RESULTS

Antibody affinity for the *N*-14 peptide

The investigated guinea pig antibody GABA$_C$ Ab *N*-14 was directed against the *N*-14 region of the human ρ1 subunit of the GABA$_C$ receptor. To assess reactivity of the affinity-purified antibody, we conducted both plate-based (ELISA) and membrane-based (dot-blotting) experiments using *N*-14 as a target. ELISA (antibody dilutions: 1/5,000 – 1/150,000) was used to determine the titer of GABA$_C$ Ab *N*-14 to *N*-14. Upon reaction with *N*-14 coated wells, GABA$_C$ Ab *N*-14 dilutions of 1/5,000 to 1/60,000 yielded absorbances above background, i.e., at least twice those determined for control wells (neutravidin only) (Fig. 1A). Higher dilutions (1/80,000 – 1/150,000) exhibited near-background absorbance. At no dilution was there observable binding to wells coated with the unrelated peptide. Antibody affinity was further tested by dot-blotting, using *N*-14 dotted on a membrane and probed with either GABA$_C$ Ab *N*-14 followed by secondary antibody, or with secondary antibody only. The peptide dots yielded a strong signal when probed with GABA$_C$ Ab *N*-14 (Fig. 1B, **lane 1**), but not when GABA$_C$ Ab N-14 was absent (**lane 2**).

Antibody affinity for ρ1 GABA$_C$

To test GABA$_C$ Ab *N*-14 reactivity with cell line-expressed ρ1 GABA$_C$ receptors, we performed Western blots using GABA$_C$-expressing SHp5-ρ1 cells and control SHSY5Y cells (Fig. 2A), as well as oocyte membrane protein preparations (Fig. 2B). Samples from SHp5-ρ1 and from ρ1 GABA$_C$-expressing oocytes probed with GABA$_C$ Ab *N*-14 followed by secondary antibody exhibited a prominent band at ~55 kDa, the expected molecular weight of human GABA$_C$ ρ1 subunit (Fig. 2A,B, **Lane 1**). This band was absent in cells lacking GABA$_C$ receptors (Fig. 2A,B, **Lane 2**). Either omission of GABA$_C$ Ab *N*-14 or pre-absorption of GABA$_C$ Ab *N*-14 led to loss of the ~55 kDa band (Fig. 2A,B, **Lanes 3–4**).

To examine the selectivity of GABA$_C$ Ab *N*-14 for GABA receptor subtypes, we analyzed, by Western blot, reactivity of the antibody with membrane protein preparations from oocytes expressing ρ1 GABA$_C$, ρ2 GABA$_C$, or α1β2γ2 GABA$_A$ receptors. Figure 2C, **section 1** shows that the ~55 kDa band, present for ρ1 GABA$_C$ (**lane c**), was absent from GABA$_A$ (**a**) and from the non-expressing control (**b**). Figure 2C, **section 2** shows that membrane preparations from human ρ2 GABA$_C$-expressing oocytes did not react with GABA$_C$ Ab *N*-14, as evidenced by the absence of a band in **lane c**. However, ρ2 GABA$_C$ was detectable by anti-ρ2 antibody (**section 3, lane c**). Thus, GABA$_C$ Ab *N*-14 immunoreacts with the GABA$_C$ ρ1 subunit, but not with either GABA$_C$ ρ2 subunit or GABA$_A$ receptors.

Western blotting was used to assess the reactivity of GABA$_C$ Ab *N*-14 for ρ1 GABA$_C$ expressed in brain and retinal tissue. Samples prepared from rat retina and rat brain, probed with GABA$_C$ Ab *N*-14, exhibited prominent bands at ~55 kDa (Fig. 2D, **section 1**). Consistent with data obtained in cell line preparations, both exclusion of GABA$_C$ Ab *N*-14 (Fig. 2D, **section 2**) and pre-absorption of GABA$_C$ Ab *N*-14 with *N*-14 (Fig. 2D, **section 3**) resulted in loss of the ~55 kDa band. Furthermore, in preparations of human and baboon retina probed with GABA$_C$ Ab *N*-14, the main observed bands were ~ 55kDa, consistent with GABA$_C$ ρ1 (Fig. 2E). Minor bands present in the Western blots can be explained by protein degradation of ρ1, and are consistent with previously-described patterns (Adamian et al., 2009; Wang et al., 2007). Thus, Western blotting with GABA$_C$ Ab *N*-14 specifically detects native GABA$_C$ ρ1 receptors from human, baboon and rat.

Interaction of GABA$_C$ Ab *N*-14 with ρ1 GABA$_C$ receptors expressed in intact cells

Incubation of ρ1 GABA$_C$-expressing oocytes with GABA$_C$ Ab *N*-14 yielded fluorescence staining of the surface membrane (Fig. 3A, **panel 1**). No labeling occurred when the oocyte was incubated either without primary antibody (**panel 2**), or when GABA$_C$ Ab *N*-14 was pre-absorbed with cognate peptide (**panel 3**). Neither non-expressing oocytes nor oocytes expressing α1β2γ2 GABA$_A$ receptors exhibited significant labeling under any of these conditions (not shown). However, oocytes expressing α1β2γ2 GABA$_A$ receptors showed surface fluorescence when probed with anti-α1 antibody (not shown). GABA$_C$ Ab *N*-14 labeling is thus specific for oocytes expressing ρ1 GABA$_C$ receptors, and cognate peptide competes with labeling.

In ρ1 GABA$_C$-expressing oocytes, we assessed the effect of GABA$_C$ Ab *N*-14 binding by electrophysiological recording of GABA-elicited responses. Waveforms in Figure 3A show responses to 3 µM GABA obtained from ρ1 GABA$_C$-expressing oocytes that were untreated, treated with GABA$_C$ Ab *N*-14 only, or treated with GABA$_C$ Ab *N*-14 and secondary antibody. Responses obtained from untreated oocytes (**black trace**) were similar to those recorded from oocytes treated with GABA$_C$ Ab *N*-14 only (**red**), or with GABA$_C$ Ab *N*-14 and secondary antibody (**green**). Peak amplitudes (mean ± SD) of responses elicited by 3 µM GABA were 3770 ± 596 nA (n=5) for untreated oocytes, 3650 ± 212 nA (n=2) for oocytes treated with GABA$_C$ Ab *N*-14 only, and 3892 ± 324 nA (n=4) for oocytes treated with GABA$_C$ Ab *N*-14 and secondary antibody. These data indicate that, with 3 µM GABA, there was no significant difference in peak amplitudes determined for untreated *vs*. GABA$_C$ Ab *N*-14 only-treated oocytes *vs*. oocytes treated with GABA$_C$ Ab *N*-14 followed by secondary antibody [ANOVA, $F_{(2, 11)}$=0.19, p=0.83]. Thus, under the investigated conditions, oocyte labeling with GABA$_C$ Ab *N*-14, alone or followed by secondary antibody, does not significantly affect the GABA-elicited response.

To further examine the interaction of GABA$_C$ Ab *N*-14 with ρ1 GABA$_C$-expressing cells, we employed GABA$_C$ Ab *N*-14 for flow cytometry analysis of SHp5-ρ1 neuroblastoma cells; non-expressing SHSY5Y cells served as controls. Probing the GABA$_C$-expressing cells with GABA$_C$ Ab *N*-14 (1/25, 1/50 and 1/1000 dilution) produced a rightward shift in mean fluorescence intensity (MFI), corresponding with the presence of ~47 to 63% positive cells (Figs. 4A,B, **green, pink and blue traces**, respectively). Neither SHp5-ρ1 cells probed with non-immune guinea pig IgG as a primary antibody (Fig. 4A, **purple**) nor non-GABA$_C$ expressing SHSY5Y cells probed with GABA$_C$ Ab *N*-14 (Fig. 4B, **purple**) showed a significant shift in MFI.

Enriched populations of GABA$_C$-expressing neuroblastoma cells recovered from flow cytometry were cultured for examination in immunofluorescence and electrophysiological experiments. Fig. 3B, **panels 1–3**, show results obtained on treatment of these GABA$_C$-expressing cells and non-expressing controls with GABA$_C$ Ab *N*-14 followed by biotinylated secondary antibody and SA-qdots, or with biotinylated secondary and SA-qdots only. These data indicate that labeling depended on both ρ1 GABA$_C$ expression and treatment with GABA$_C$ Ab *N*-14. The waveforms show electrophysiological results obtained from SHp5-ρ1 cells that were untreated, treated with GABA$_C$ Ab *N*-14 only, or treated with GABA$_C$ Ab *N*-14 followed by biotinylated secondary antibody and SA-qdots. Presentation of 10 µM GABA under these three conditions elicited responses of similar peak amplitude and kinetics (**black, red and green traces**, respectively). Peak amplitudes of GABA-elicited responses determined in multiple experiments were 1736 ± 284 pA (n=9) for untreated cells, 1851 ± 278 pA (n=8) for cells treated with GABA$_C$ Ab *N*-14 only, and 1739 ± 319 pA (n=10) for cells treated with GABA$_C$ Ab *N*-14, biotinylated secondary antibody and SA-qdots, and there was no significant difference among the three groups of peak amplitude data [ANOVA, $F_{(2, 27)}$=0.04, p=0.96]. Thus, as with ρ1 GABA$_C$-expressing oocytes,

treatment of ρ1 GABA$_C$-expressing neuroblastoma cells with GABA$_C$ Ab *N*-14 yields robust immunolabeling and no significant perturbation of electrophysiological activity.

Interaction of GABA$_C$ Ab *N*-14 with human retina

GABA$_C$ Ab *N*-14 treatment of tissue sections prepared from human retina yielded fluorescence staining primarily within the inner plexiform layer (Fig. 5, **panel 1**). No significant labeling occurred when the slice was incubated either without primary antibody (**panel 2**), or when GABA$_C$ Ab *N*-14 was pre-absorbed with cognate peptide (**panel 3**). Labeling by GABA$_C$ Ab *N*-14 is thus relatively specific for the inner plexiform layer of human retina.

DISCUSSION

The present study reports the characterization of a novel polyclonal antibody, GABA$_C$ Ab *N*-14, that targets the human ρ1 GABA$_C$ receptor. This antibody differs in several respects from a polyclonal anti-ρ antibody originally described by Enz et al. (1996) and used in multiple subsequent studies (e.g., Picaud et al., 1998; Pattnaik et al., 2000; McCall et al., 2002; Klooster et al., 2004). Preparation of the previously described antibody employed, as an immunogen, a large recombinant fusion peptide that includes amino acids 1–171 of the mature rat ρ1 sequence. By contrast, the antigen used for the presently investigated antibody was a short peptide that contains amino acids 23–36 of the mature human ρ1 sequence, and that was conjugated to KLH. Furthermore, the polyclonal antibody generated by Enz et al. (1996) recognized ρ2 and ρ3 in addition to the ρ1 subunit, perhaps as a consequence of the large size of the recombinant peptide immunogen used and, thus, a higher likelihood of this immunogen's sequence homology with multiple subunit types. By contrast, as elaborated below, the present antibody has high affinity and specificity for the ρ1 subunit, and does not cross-react with other GABA$_C$ subunits.

Specificity and reactivity of GABA$_C$ Ab *N*-14

The results provide several lines of evidence for a high specificity and high affinity of GABA$_C$ Ab *N*-14 for the human ρ1 GABA$_C$ subunit. First, ELISA data indicate robust binding of GABA$_C$ Ab *N*-14 to epitopes displayed by the *N*-14 peptide (Fig. 1). Second, for ρ1 GABA$_C$-expressing cells, Western blot analyses indicate predominant immunoreactivity of GABA$_C$ Ab *N*-14 with a single protein of MW ~55 kDa, the expected molecular weight of the ρ1 subunit (Fig. 2). Third, the antibody exhibits robust immunostaining of cells expressing ρ1 GABA$_C$ receptor (Fig. 3A,B). Fourth, flow cytometry reveals a rightward shift in the MFI of ρ1 GABA$_C$-expressing neuroblastoma cells probed with GABA$_C$ Ab *N*-14 (Fig. 4). Fifth, human retina probed with GABA$_C$ Ab *N*-14 shows immunolabeling primarily within the inner plexiform layer, and this labeling is suppressed by both pre-absorption of GABA$_C$ Ab *N*-14 with the cognate peptide, and the omission of GABA$_C$ Ab *N*-14 (Fig. 5). The inner plexiform layer includes the axonal region of bipolar cells, a known primary locus of GABA$_C$ receptors (Feigenspan et al., 1993; Qian and Dowling, 1993; Qian et al., 1997).

The reactivity of GABA$_C$ Ab *N*-14 with GABA$_C$-expressing cells raises the interesting question of whether binding of this antibody preserves the receptor's physiological activity. The waveforms of Figs. 3 A,B and accompanying text, which indicate the absence of an effect on the GABA-elicited response under conditions of robust immunological staining, are consistent with this possibility. However, a perturbing action by receptor-bound antibody is not ruled out, as the evident immunostaining signal (Figs. 3A,B) could reflect antibody binding to only a minor fraction of the overall receptor population. A previous study describing an antibody raised against the *N*-terminal half of recombinant ρ1 subunit reported

an inhibitory effect on GABA-elicited responses in ρ1 GABA$_C$-expressing oocytes (Ekema et al., 2001).

Based on an antibody concentration of 0.24 mg/ml in the affinity-purified GABA$_C$ Ab *N*-14 preparation (see Material and Methods), dilutions used in the present experiments correspond with antibody concentrations ranging from 0.048 – 0.004 µg/mL in ELISA, 0.24 – 9.6 µg/mL in flow cytometry, 0.12 – 0.24 µg/mL in immunofluorescence labeling, and 0.024 µg/mL in Western blot. Such concentrations compare favorably with typical ranges of polyclonal antibody concentrations used in ELISA (~ 0.1 µg/mL), flow cytometry (~ 1 µg/mL), Western-blot (~ 1 µg/mL) and immunofluorescence labeling of cells (~ 5 µg/mL), indicating a high sensitivity of GABA$_C$ Ab *N*-14 for its target. GABA$_C$ Ab *N*-14 antibody, directed against the unstructured region in the *N*-terminal extracellular domain of the human GABA$_C$ ρ1 subunit, thus joins a group of other polyclonal antibodies with reactivity at the extracellular domains of the cys-loop and other ligand-gated ion channel receptors.

Conclusion

GABA$_C$ Ab *N*-14, a novel polyclonal antibody directed against a peptide in the N-terminal extracellular domain of the human ρ1 subunit of GABA$_C$ receptors, exhibits robust binding activity to ρ1 as determined by Western blot, flow cytometry and immunostaining. It furthermore shows affinity for the inner plexiform layer of human retina, a known locus of ρ1-containing GABA$_C$. The present antibody will be valuable for future investigations of ρ1 expression and localization in retina and other neural tissues. It may also have application in fundamental and clinically oriented studies as a ρ1-targeting component for delivering biomolecules to GABA$_C$ receptors.

Research highlights

- GABA$_C$ Ab *N*-14 is a new polyclonal antibody directed against the ρ1 GABA$_C$ subunit.

- The antibody binds specifically to ρ1 expressed in oocytes and neuroblastoma cells.

- Antibody binding preserves electrophysiological activity of the target GABA$_C$.

- In human retina sections, the antibody preferentially labels inner plexiform layer.

Acknowledgments

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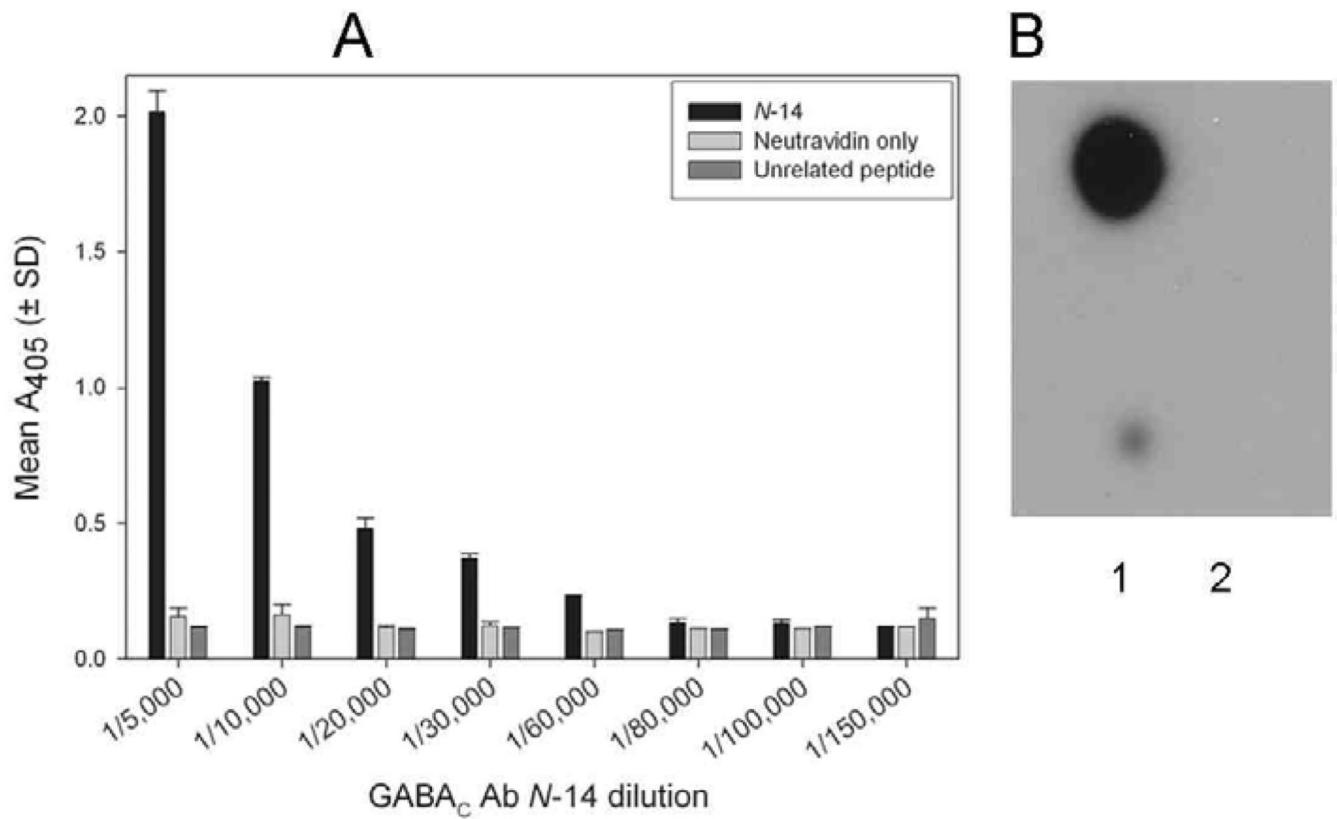
Fig. 1.
Reactivity of GABA$_C$ Ab N-14 with N-14. **A**: Titration of GABA$_C$ Ab N-14 by ELISA.
GABA$_C$ Ab N-14 was tested at the indicated dilutions for reactivity with biotinylated N-14
(black bars), unrelated biotinylated peptide (dark gray bars), or no peptide (i.e., neutravidin
only; light gray bars). Absorbance values (405 nm) are means (± SD) of duplicate
measurements. **B:** Dot-blot assay using 1 ng (top) and 0.1 ng (bottom) of N-14 probed with
GABA$_C$ Ab N-14 (lane 1) or with secondary antibody only (lane 2).
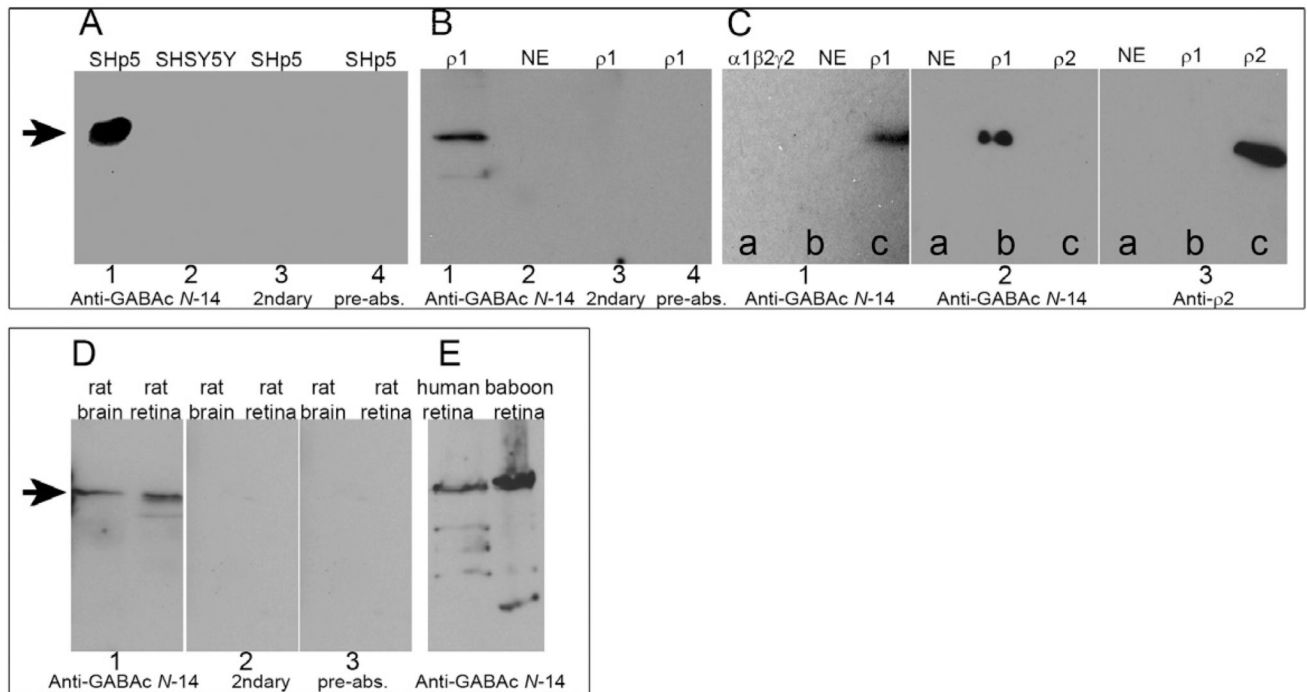
Fig. 2.
Western blots. **A:** Whole-cell lysates of GABA$_C$-expressing SHp5-ρ1 cells and non-GABA$_C$ expressing SHSY5Y cells. Lane 1: SHp5-ρ1 probed with GABA$_C$ Ab *N*-14. Lane 2: SHSY5Y probed with GABA$_C$ Ab *N*-14. Lane 3: SHp5-ρ1 probed with secondary antibody only. Lane 4: SHp5-ρ1 probed with GABA$_C$ Ab *N*-14 pre-absorbed with *N*-14. **B:** Oocyte membrane preparations. Lanes 1, 3 and 4: GABA$_C$ ρ1 expressing oocytes. Lane 2: Non-expressing control oocyte. Experimental conditions used for lanes 1–4 are otherwise identical, respectively, to lanes 1–4 in A. **C:** Oocyte membrane preparations. Section 1: Preparations of α1β2γ2 GABA$_A$-expressing oocytes (lane a), non-expressing oocytes (b), and ρ1 GABA$_C$-expressing oocytes (c) probed with GABA$_C$ Ab *N*-14. Section 2: Preparations of non-expressing oocytes (lane a), ρ1 GABA$_C$-expressing oocytes (b) and human ρ2 GABA$_C$-expressing oocytes (c) probed with GABA$_C$ Ab *N*-14. Section 3: Same preparations as those of section 2, probed with anti-ρ2 antibody. **D**: Whole cell lysates of rat brain and rat retina probed with: GABA$_C$ Ab *N*-14 (Section 1), secondary antibody only (Section 2), or GABA$_C$ Ab *N*-14 pre-absorbed with *N*-14 (Section 3). **E**: Whole cell lysates of human and baboon retina, probed with GABA$_C$ Ab *N*-14. On all blots, the arrows indicate MW ~ 55 kDa.
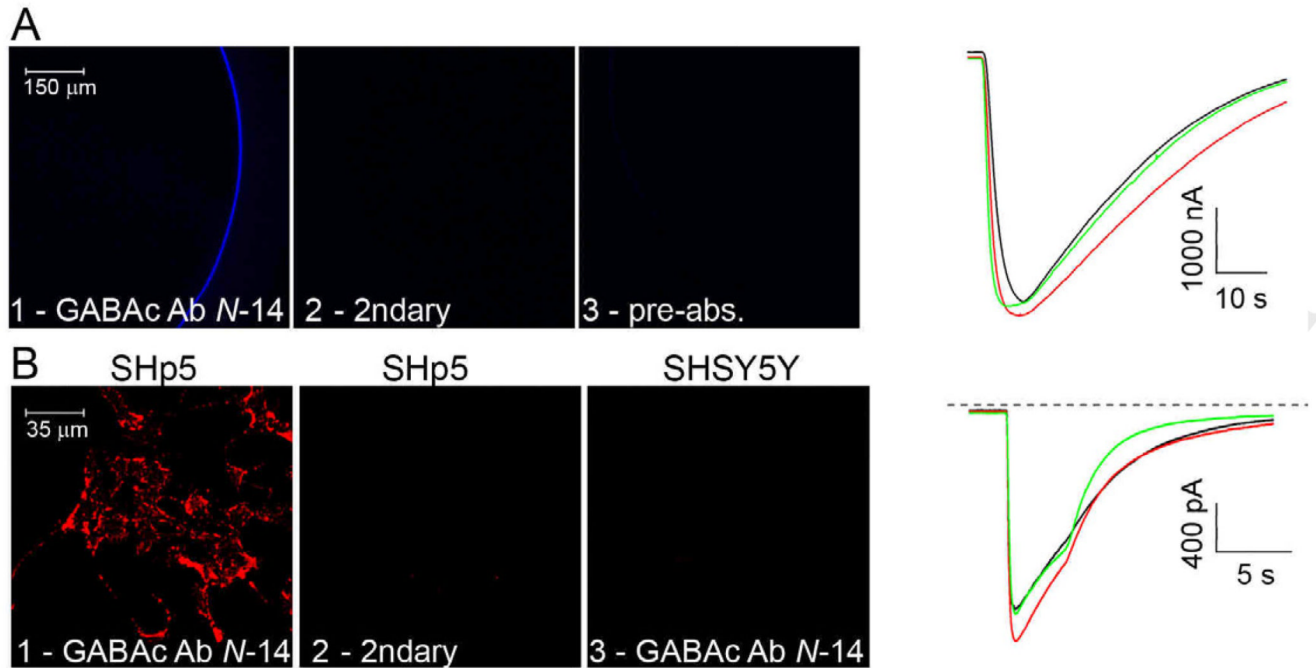
Fig. 3.
Cell immunostaining and electrophysiology. **A:** Fluorescence images of ρ1 GABA$_C$-expressing oocytes, following treatment with GABA$_C$ Ab *N*-14 followed by Cy5-conjugated secondary (<u>1</u>), with secondary antibody only (<u>2</u>), or with pre-absorbed GABA$_C$ Ab *N*-14 followed by secondary antibody (<u>3</u>). *Right:* Membrane current responses of ρ1-expressing oocytes elicited by 3 µM GABA. Untreated oocyte (black trace); oocyte treated with GABA$_C$ Ab *N*-14 only (red); and oocyte treated with GABA$_C$ Ab *N*-14 and secondary antibody (green). **B**: Fluorescence images of neuroblastoma cells. (<u>1</u>): SHp5-ρ1 cells incubated with GABA$_C$ Ab *N*-14, followed with biotinylated secondary antibody and SA-qdots; (<u>2</u>): SHp5-ρ1 cells incubated without GABA$_C$ Ab *N*-14, but with biotinylated secondary antibody and SA-qdots; (<u>3</u>): SHSY5Y incubated in conditions similar to those of image <u>1</u>. *Right:* Membrane current responses of SHp5-ρ1 cells to 10 µM GABA (holding potential = − 60 mV). Untreated cell (black trace); cell treated with GABA$_C$ Ab *N*-14 only (red); and cell treated with GABA$_C$ Ab *N*-14, biotinylated secondary antibody, and SA-qdots (green).
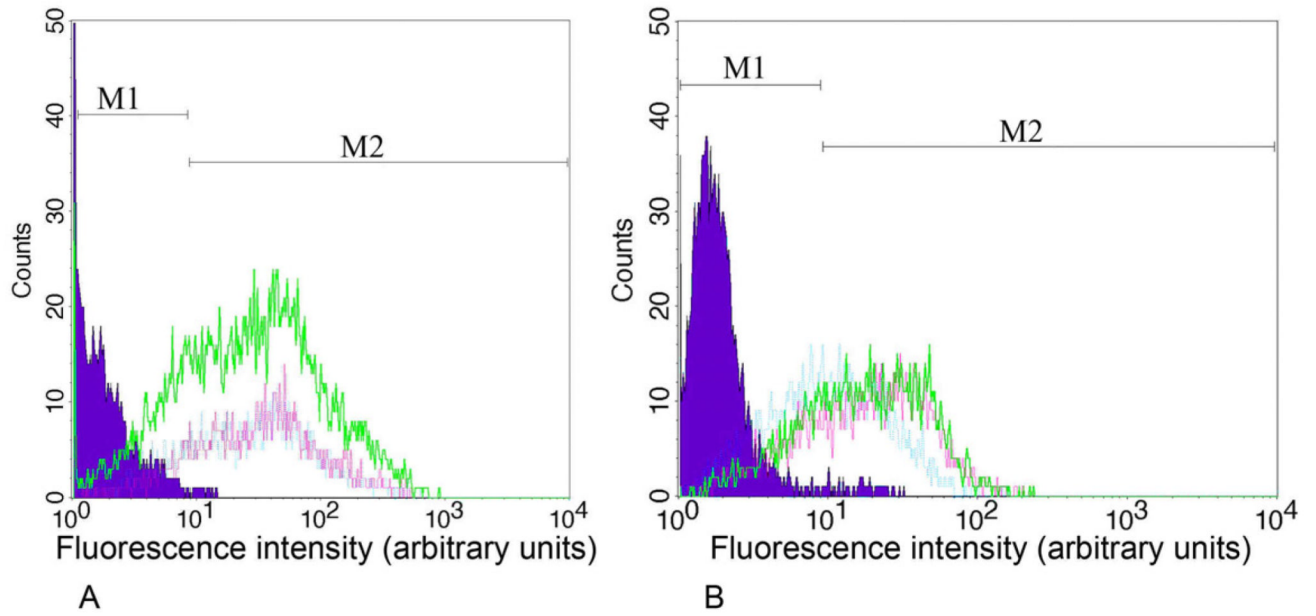
Fig. 4.
Flow cytometry profiles for SHp5-ρ1 and SHSY5Y cells, using $GABA_C$ Ab *N*-14 or non-immune guinea pig IgG. **A**: *Purple area*: SHp5-ρ1 cells probed with non-immune guinea pig IgG as primary antibody. *Green, pink, and blue traces*: SHp5-ρ1 cells probed with $GABA_C$ Ab *N*-14 at dilutions of 1/25, 1/50 and 1/1,000, respectively. **B**: *Purple area*: SHSY5Y cells probed with $GABA_C$ Ab *N*-14 as a primary antibody. *Green, pink* and *blue traces*: SHp5-ρ1 cells probed with $GABA_C$ Ab *N*-14 at dilutions identical to those of A. Regions M1 and M2: background (i.e., unstained) cells and cells with positive staining, respectively.
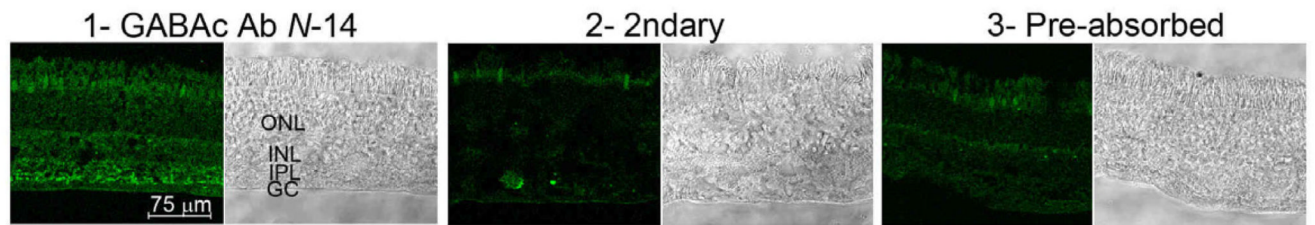
Fig. 5.
Fluorescence and brightfield images of human retina cryosections, treated with GABA$_C$ Ab *N*-14 followed by FITC-conjugated secondary (1); with secondary antibody only (2); or with pre-absorbed GABA$_C$ Ab *N*-14 followed by secondary antibody (3). Labels in brightfield image (1) approximate the regions of the ganglion cell layer (GC), inner plexiform layer (IPL), inner nuclear layer (INL), and outer nuclear layer (ONL).