GABA Receptor $\rho_1$ Subunit Interacts with a Novel Splice Variant of the Glycine Transporter, GLYT-1*

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Ionotropic $\gamma$-aminobutyric acid (GABA$_\gamma$ and GABA$_C$) receptors mediate fast synaptic inhibition in the central nervous system. GABA$_\gamma$ receptors are expressed predominantly in the retina on bipolar cell axon terminals, and are thought to mediate feedback inhibition from GABAergic amacrine cells. Utilizing the yeast two-hybrid system, we previously identified MAP1B as a binding partner of the GABA$_\gamma$ receptor $\rho_1$ subunit. Here we describe the isolation of an additional $\rho_1$ interacting protein: a novel C-terminal variant of the glycine transporter GLYT-1. We show that GLYT-1 exists as four alternatively spliced mRNAs which encode proteins expressing one of two possible intracellular N- and C-terminal domains. Variants containing the novel C terminus efficiently transport glycine when expressed in COS cells, but with unusual kinetics. We have confirmed the interaction between the novel C terminus and $\rho_1$ subunit and demonstrated binding in heterologous cells. This interaction may be crucial for the integration of GABAergic and glycineergic neurotransmission in the retina.

In the central nervous system, fast inhibitory neurotransmission is mediated predominantly by the activation of ionotropic $\gamma$-aminobutyric acid (GABA)$^1$ receptors and glycine receptors localized to synaptic sites. The synaptic activity of these transmitters is terminated by sodium-dependent transport into neurons or glial cells in the vicinity of the synapse. Two categories of ionotropic GABA receptor are expressed in the central nervous system: GABA$_\gamma$ and GABA$_C$ receptors (1–4). Both are GABA-gated chloride channels, but are classified as different subtypes because of their distinct pharmacological and biochemical properties, as well as expression patterns. GABA$_\gamma$ receptors are expressed throughout the central nervous system (1, 2), whereas GABA$_C$ receptors are expressed predominantly in the retina, with lower levels detected in other central nervous system regions (5–7). GABA$_C$ receptors are enriched on bipolar cell axon terminals where they receive GABAergic input from amacrine cells (8, 9). Activation of these receptors inhibits glutamate release onto retinal ganglion cells (9–11), and is likely to be important in tuning the temporal resolution and spatial contrast of responses to visual stimuli in the retina (12).

GABA$_\gamma$ receptors are thought to be formed from $\rho$ subunits which are localized to retinal bipolar cells (13). $\rho$ Subunits expressed in Xenopus oocytes exhibit the same pharmacological and electrophysiological properties as GABA$_C$ receptors studied in the retina (8, 14, 15). Three $\rho$ subunits have been cloned, and each forms functional homo-oligomeric receptors when expressed in Xenopus oocytes (12, 13). However, there is pharmacological and biochemical evidence to suggest that $\rho_1$ and $\rho_2$ subunits can also form functional hetero-oligomers (16, 17). In contrast, GABA$_\gamma$ receptors are more complex hetero-oligomers, and are formed from $\alpha$ (1–6), $\beta$ (1–3), $\gamma$ (1–3), $\delta$, $\epsilon$, and $\pi$ subunits, with the majority of GABA$_\gamma$ receptors comprising at least one $\alpha$, one $\beta$, and one $\gamma$ subunit (2).

Although ligand-gated ion channels expressed in heterologous, non-synaptic systems display essentially native electrophysiological and pharmacological properties, it is becoming increasingly clear that they do not exist simply as isolated macromolecules at synaptic membranes in vivo. Yeast two-hybrid screens using intracellular domains of ionotropic glutamate receptor subunits as baits, have isolated various proteins as binding partners for these receptors. These proteins may function to maintain the clustered distribution of the receptor at synaptic sites, or for bringing other important proteins into close proximity for intracellular signaling up- or downstream from the receptor (18–20). These studies show that the post-synaptic density at excitatory synapses is a complex array of proteins which interact with ionotropic glutamate receptors, either directly or via scaffolding proteins (20).

More recently, similar studies aimed at understanding the architecture of the inhibitory synapse have used the large intracellular loop between transmembrane regions (TM) three and four of ionotropic GABA receptor subunits as yeast two-hybrid baits. These studies have identified a novel tubulin-binding protein, GABARAP, which interacts with the $\gamma_2$ subunit of GABA$_\gamma$ receptors and may function to localize the receptor at synaptic sites (21). Similarly, GABA$_C$ receptors have been shown to interact with MAP1B, a microtubule-associated protein which can influence the subcellular distribution of the receptor (22).

To investigate further the identity of GABA$_C$ receptor interacting proteins, we have used the $\rho_1$ subunit intracellular loop between TM3 and TM4 as a yeast two-hybrid bait for additional screening of a retinal library. Here we report that a novel C-terminal splice variant of the glycine transporter GLYT-1 interacts specifically with the $\rho_1$ subunit bait and show that

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The abbreviations used are: GABA, $\gamma$-aminobutyric acid; GLYT, glycine transporter type 1; TM, transmembrane region; MAF, microtubule-associated protein; a.a., amino acid; GST, glutathione S-transferase; PKA, cAMP-dependent protein kinase; NMDA, N-methyl-D-aspartate; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s).
this interaction occurs in a cellular environment. We describe the tissue distribution and functional characterization of GLYT-1 variants isolated from the retina. Finally, we propose a role for co-localization of these two proteins in the integration of GABA and glycineric signaling, and the modulation of glutamatergic neurotransmission at bipolar cell-retinal ganglion cells synapses. This is the first report to demonstrate a direct protein-protein interaction between a neurotransmitter receptor and a neurotransmitter transporter.

**EXPERIMENTAL METHODS**

**Yeast Two-hybrid System**—Approximately 3 million clones from a bovine retinal cDNA library constructed in the Gal4 activation domain-carrying vector pACTII (CLONTECH) were screened with a bait encoding the intracellular domain of the human \( \rho_1 \) subunit cloned in the Gal4-DNA-binding domain-carrying vector pAS2-1 (CLONTECH), as described previously (22). \( \rho_1 \), \( \rho_2 \), and GABA\(_{\text{B}}\) receptor intracellular domain bait plasmids were constructed by PCR amplification of cDNA fragments, followed by in-frame ligation into pAS2-1, as described previously (22).

**Isolation of bGLYT-1 cDNA Clones**—DNA sequencing was carried out on both strands using Sequenase\textsuperscript{TM} Version 2.0 (U. S. Biochemical Corp.). The insert of “clone 6” isolated from the yeast-two hybrid screen was \( \Phi X 174 \) Plabeled and used to screen an adult bovine retinal cDNA library (Stratagene) by hybridization as described by Blondel et al. (23). A single clone, designated \( \lambda C 6 p A 1 \), was obtained that contained a 1779-bp open reading frame similar to GLYT-1, followed by 96 bp of 3’-untranslated sequence and a poly(A) tail. To determine if the bovine retina expressed GLYT-1 variants containing the previously characterized C terminus (D, Fig. 2) retinal cDNA using a bovine specific upstream primer located in the core region (GenBank U52689) 5’-CAGC-CATTGTGAGTAGGATG-3’, and a GLYT-1 downstream primer to a region of 3’-untranslated sequence common to human and mouse (GenBank X67056) 5’-TCATACTGGGGATCTGGTGGAA-3’. Amplification was using standard conditions (24) and PCR products were blunt-end ligated, subcloned to the BstEII site of pGEM-4Z (Promega), and sequenced. All clones contained a 667-bp insert encoding the bovine homologue of the mGLYT-1 C terminus. Additional 5’ end sequences were isolated by ligation anchor (LA-) PCR of bovine retinal cDNA as described by Trout et al. (25), using a bGLYT-1 downstream primer (GenBank U52689) 5’-GGATGTCGAGAT-GACCAAGTGTTGACTA-3’, and an upstream primer specific to the anchor sequence of bovine retinal cDNA was amplified, blunt-end ligated into the HincII site of pGEM-3Z, and sequenced. Sequences were confirmed in two independent LA-PCR experiments.

**Expression Pattern of GLYT-1 Variants by RT-PCR**—Expression of mRNA encoding GLYT-1 variants in retina, kidney, hypothalamus, or adrenal was examined by RT-PCR using specific primer pairs. These primers amplified a sequence common to all forms of bGLYT-1 (univer-

al primers), or were specific for transcripts containing N termini “A” or “B”, or C termini “D” or “F,” where one primer of a pair was specific for a variant, and the other primer used was to sequence in the “core” region (see Fig. 2). The up- and downstream primer sequences (5’ to 3’) and the length of their products (in bp) are: universal (210) 5’-CTGTGC-TCATATCCGGGAGTCCTGGAA-3’. Amplification was using standard conditions (24) and PCR products were blunt-end ligated, subcloned to the BstEII site of pGEM-4Z (Promega), and sequenced. All clones contained a 667-bp insert encoding the bovine homologue of the mGLYT-1 C terminus.
c-terminal of gion corresponding to the N-terminal and core regions (28). The identity to the mouse glycine transporter mGLYT-1A in a re-deduced amino acid (a.a.) sequence of this clone showed 94.9%

To determine if the interaction between clone 6 and r

tem. Fig. 1

range of GABA receptor subunits in the yeast two-hybrid sys-

units, we tested clone 6 against intracellular domains from a

MAP1B (22), we isolated a novel clone, termed clone 6 (Fig. 1

proteins. In addition to the microtubule-associated protein

Western blotting with anti-Myc 9E10.

anti-GLYT-1E/F, or nonimmune IgG. Bound proteins were detected by

COS cells were lysed, and treated as described above using 5

and detected by autoradiography.

After washing four times in lysis buffer, the first two washes supple-

lysate was incubated with protein A conjugated to Sepharose beads for

used ligation anchor PCR of bovine retinal cDNA to ensure we

protein A beads plus 5

of anti-GLYT-1E/F antibody for 1 h at 4 °C .

1 h at 4 °C to remove nonspecific binding, followed by incubation with protein A beads plus 5 μg of anti-GLYT-1E/F antibody for 1 h at 4 °C. After washing four times in lysis buffer, the first two washes supplemented with 0.5 mM NaCl, bound proteins were separated by SDS-PAGE, and detected by autoradiography.

-Co-immunoprecipitation—After overnight expression, co-transfected COS cells were lysed, and treated as described above using 5 μg of anti-GLYT-1E/F, or nonimmune IgG. Bound proteins were detected by Western blotting with anti-Myc 9E10.

RESULTS

Isolation of a Novel GLYT-1 Variant as a p1 Subunit Binding Partner by Yeast Two-hybrid Screening—Using the yeast two-

hybrid system with the large intracellular domain between TM3 and TM4 of the p1 subunit as a bait we screened a bovine retinal cDNA library to identify GABA_\text{C} receptor interacting proteins. In addition to the microtubule-associated protein MAP1B (22), we isolated a novel clone, termed clone 6 (Fig. 1A).

To determine if the interaction between clone 6 and p1 is specific, or a general feature of ionicotropic GABA receptor sub-

units, we tested clone 6 against intracellular domains from a range of GABA receptor subunits in the yeast two-hybrid system. Fig. 1B shows that the interaction is specific to the GABA_\text{C} receptor p1 subunit, since no interaction is seen with GABA_\text{A} receptor subunits nor the GABA_\text{B} receptor subunits due to the presence of a C-terminal domain variant of GLYT-1.

Hybridization screening of an adult bovine retinal cDNA library with a \(^{32}\text{P}\)-labeled insert from clone 6 isolated a clone containing a 1779-bp open reading frame, termed C6pA1. The deduced amino acid (a.a.) sequence of this clone showed 94.9% identity to the mouse glycine transporter mGLYT-1A in a region corresponding to the N-terminal and core regions (28). The C-terminal of C6pA1, identical in sequence to clone 6, shares only 10% a.a. identity with the mGLYT-1 C terminus. Taken together these data indicate that C6pA1 is a bovine homolog of GLYT-1 with a novel C terminus (Fig. 2). Bovine retinal cDNA clones containing a C terminus homologous to mGLYT-1, 93.1% identity, were obtained by RT-PCR. Since clone C6pA1 contained only a few bases of 5′-untranslated sequence, we used ligation anchor PCR of bovine retinal cDNA to ensure we had isolated the complete open reading frame. We identified three species of mRNA encoding bGLYT-1 5′ ends, two encoding N terminus A with different 5′-untranslated regions, and one encoding N terminus B. The complexity of bGLYT-1 5′ splice products is similar to that reported for the mouse GLYT-1 (29). The bovine N termini are termed A and B, according to the mGLYT-1 nomenclature, and the C termini are termed D and E. Bovine clones homologous to mGLYT-1-C, an alternatively spliced N- and C-terminal variants and the core region. N terminus A is four a.a. shorter than B, both encode intracellular domains. C terminus D is 40 a.a. longer than E and contains a 12th transmembrane spanning domain, whereas the novel C terminus E does not.

Expression of Alternatively Spliced Forms of GLYT-1 mRNA—The tissue distribution of GLYT-1 mRNAs containing N terminus A or B, or C terminus D or E was examined by RT-PCR (Fig. 3). The overall level of GLYT-1 transcript expression, as determined using primers to the core region, was similar in adrenal, hypothalamus, kidney, and retina (Fig. 3, universal). The expression levels of N-terminal A and C-terminal D containing transcripts were also similar in all tissues. By contrast,
N-terminal B containing transcripts were present at higher levels in hypothalamus and retina than adrenal and kidney. Transcripts containing the ρ1-interacting C-terminal E (as determined by the presence of the expected 263-bp band) occurred at highest levels in the hypothalamus, but also at significant levels in the retina and kidney, and was absent in the adrenal. Additional PCR products were obtained with amplification of C-terminal E primers: the sequences of these products were not determined.

Confirmation of ρ1-GLYT-1E/F Interaction by Overlay Assay—To demonstrate a direct interaction between the ρ1 intracellular domain and GLYT-1E/F C terminus (clone 6), GST fusion proteins were constructed encoding both of these polypeptides for use in overlay assays in which no other proteins are present. GST-p1 was constructed in pGEX-2TK, which has an Arg-Arg-Ala-Ser motif engineered just C-terminal to GST, allowing in vitro labeling of the fusion protein with [γ-32P]ATP by the catalytic subunit of PKA. Gel overlay assays indicated that [32P]-labeled GST-p1 bound to GST-clone 6, but not to GST alone, demonstrating that ρ1 intracellular domain binds directly to GLYT-1E/F C terminus, and no further factor is required for the interaction (Fig. 4).

Functional Characterization of Different Forms of GLYT-1—Constructs encoding GLYT-1A, -1B, -1E, and -1F were transfected into COS cells and the kinetic and pharmacological characteristics of glycine uptake measured (Fig. 5). Variation in the intracellular N-terminal domain had no significant effect on the kinetic properties of GLYT-1, as demonstrated by the similar properties of GLYT-1A compared with B, and of GLYT-1E compared with F (data not shown). However, variation in the C-terminal domain has a dramatic effect on the kinetic properties of the expressed transporter. GLYT-1B has a $K_m$ for glycine of 59 ± 8 μM and a $V_{max}$ of 4.7 ± 0.5 nmol/mg/ min. In contrast, GLYT-1F showed complex nonlinear kinetics which cannot be fit using a single Michaelis-Menten equation. A $K_m$ could not be reliably estimated for GLYT-1F, yet it had an estimated $V_{max}$ of 1.8 ± 0.2 nmol/mg/min. The time course of 10 μM [3H]glycine uptake by GLYT-1B was linear for the first 10 min, whereas uptake by GLYT-1F was linear over at least 1 h. The average initial velocity of glycine uptake was 95 ± 10 pmol/mg/min by cells expressing GLYT-1B and 24 ± 4 pmol/mg/min for GLYT-1F. Although variation in the C-terminal of GLYT-1 affects kinetic properties, it does not alter the sodium or chloride ion dependence of glycine uptake by GLYT-1E/F. No specific uptake was detected when sodium was substituted with either choline or NMG, or when chloride was substituted with acetate or gluconate. Nor do the pharmacological properties differ between bGLYT-1 variants. Specific uptake of radiolabeled glycine by all four variants was inhibited 85–90% by unlabeled glycine and sarcosine, whereas L-alanine, GABA, and MeAIB had no effect. These data are consistent with the properties of other GLYT-1 transporters. Proline, reportedly an inhibitor of the human GLYT-1 (25), has no effect on bovine GLYT-1 variants.

Construction and Characterization of Anti-GLYT-1E/F-specific Antibodies—Polyclonal antibodies were raised against a GST fusion protein of clone 6 (GLYT-1E/F C-terminal intracellular domain) and tested for specificity in a number of assays. COS cells transfected with a construct encoding full-length GLYT-1E were prepared for immunofluorescence and stained with GST-1E/F antiserum. Fig. 6A shows some plasma membrane and predominantly endoplasmic reticulum staining in permeabilized GLYT-1E transfected cells, but not in untransfected cells. The staining was abolished by pre-absorption of the antiserum with GST-clone 6, the polypeptide used to raise the antibodies. No signal was detected in non-permeabilized transfected cells stained with anti-GLYT-1E/F, suggesting that the C terminus is intracellular and not accessible to the anti-
shows a 60-kDa band present in anti-Myc 9E10. Western blotting analysis using anti-Myc 9E10 nates, due to the very high levels of protein expression in COS permeabilized cells, endoplasmic reticulum staining predominates; that is, an equivalent amount of lysate from GLYT-1E-transfected cells, but not in untransfected cells. The band was not seen when the antisera was pre-absorbed with GST-clone 6. Unfortunately, this antisera was unsuitable for Western blotting.

Interaction of p1 and GLYT-1E/F in Heterologous Cells—GST-clone 6 was also used in affinity purification (pull-down) assays to analyze its binding to full-length p1 from cell lysates. COS cells were transfected with a construct encoding full-length p1 subunit, modified by addition of the Myc epitope between residues 4 and 5 at the N terminus of the protein. Previous studies have demonstrated that this addition is functionally silent for a range of GABA_A receptor subunits (27). Staining of p1^{Myc}-transfected COS cells with anti-Myc 9E10 in both non-permeabilized and permeabilized conditions, shows that p1^{Myc} reaches the cell surface efficiently (Fig. 7A). In permeabilized cells, endoplasmic reticulum staining predominates, due to the very high levels of protein expression in COS cells. No signal was seen in untransfected cells stained with anti-Myc 9E10. Western blotting analysis using anti-Myc 9E10 shows a 60-kDa band present in p1^{Myc} transfected cells, that is not seen in untransfected cells (Fig. 7B). To analyze the interaction between p1^{Myc} and the GLYT-1E/F C terminus, 1% Triton X-100 extracts of p1^{Myc}-transfected COS cells were incubated with GST-clone 6 bound to glutathione-agarose beads. As a positive control, p1^{Myc} binding to GST-MAP1B (p1-binding region) is also shown (22). Fig. 7C shows that p1^{Myc} binds to GST-clone 6 as well as GST-MAP1B, but not to GST alone.

To demonstrate that both full-length proteins interact in a cellular environment, we co-expressed p1^{Myc} and GLYT-1E in COS cells. After permeabilization with 0.1% Nonidet P-40 these cells were co-stained with anti-GLYT-1E/F and anti-Myc 9E10 and analyzed by immunofluorescence. Fig. 8A demonstrates that GLYT-1E/F binding to GLYT-1E is present in anti-GLYT-1E/F immunocomplexes, but not non-immune IgG complexes. These data demonstrate that these two full-length proteins interact in COS cells.

**DISCUSSION**

We have identified a novel C-terminal variant of the sodium-dependent glycine transporter GLYT-1, which specifically interacts with the p1 subunit of GABA_A receptors in recombinant systems. Expression of GLYT-1 variants containing the novel C terminus produces glycine transporters with identical pharmacological, but different kinetic properties to GLYT-1 variants containing the previously characterized C terminus. We show that the novel C-terminal variant is expressed in the retina, the predominant site of GABA_A receptor expression. In the yeast two-hybrid system, the novel C terminus specifically interacts with the p1 subunit, showing no interaction with p2 or GABA_A receptor subunits. Gel overlay assays show that this interaction requires no other factors, and affinity purification and immunoprecipitation show the interaction occurs in a cellular environment. Thus, we provide the first evidence of a direct interaction between a neurotransmitter receptor and a neurotransmitter transporter.
proposed membrane topology of 12 TMs and intracellular N and C termini (30). Alternative splicing of the GLYT-1 N terminus has been reported for human, rat, and mouse homologs. These variants differ in their pattern of expression, but produce transporters with identical kinetic and pharmacological properties (28, 31, 32). Our results show that the same is true for bovine GLYT-1 N-terminal variants.

C-terminal splicing effects both the pattern of expression and kinetic properties of the transporter, but has no effect on either its ion dependence or pharmacology. Variants containing the novel C terminus, GLYT-1E and -1F, have complex nonlinear kinetics that cannot fit with a single Michaelis-Menten equation, whereas the kinetics of variants containing the known C terminus, GLYT-1A and -1B, are linear and fit by a single Michaelis-Menten equation. The basis for the nonlinear kinetics displayed by GLYT-1E/F is unknown, but may be due to the presence of multiple binding sites with different substrate affinities or a single site whose affinity is altered by modulators. Alternatively, this complex response could arise from a combination of inward and outward transport, where outward flux is more significant for the GLYT-1E/F variants than for GLYT-1A/B. These data also suggest that the regions of the GLYT-1 protein necessary for determining its ion dependence and pharmacological properties are located outside of the C terminus.

When compared with the previously characterized GLYT-1A/B C terminus, the novel, p1 interacting C terminus is 45 a.a. residues shorter. This C terminus shares little a.a. identity with the GLYT-1A/B C terminus and does not contain an amphipathic region corresponding to TM12. Previously it was shown that progressive truncation of the GLYT-1A/B C terminus produced a progressive decrease in transport activity (33). Expression of the largest C-terminal truncation in COS cells failed to localize transporters to the plasma membrane, and reconstitution experiments showed it was non-functional. These data suggest the C terminus is important for normal function and protein targeting. Despite the sequence divergence and apparent lack of TM12, expression of GLYT-1E/F in COS cells produces a functional glycine transporter indicating the novel C terminus provides the information necessary for function and cell surface expression. Immunocytochemistry of GLYT-1E/F transfected COS cells clearly indicates that this novel C terminus is located intracellularly, suggesting that either this C terminus crosses the plasma membrane using a non-amphipathic domain, or that the proposed topology for these transporters is incorrect.

GLYT-1E/F Interacts with p1 Subunit of GABA<sub>C</sub> Receptors—
Yeast two-hybrid studies using glutamate or GABA<sub>C</sub> receptor subunit intracellular domains as baits have identified protein interactions which mediate signaling functions and/or affect receptor localization (18–20). Previously we reported the specific interaction between GABA<sub>C</sub> receptor p1 subunit and microtubule-associated protein 1B (MAP1B), which can influence the subcellular localization of the receptor (22). In this study we show that a novel GLYT-1 C-terminal variant and the large intracellular loop of the GABA<sub>C</sub> receptor p1 subunit interact in a cellular environment but do not have a significant effect on the localization of either protein. Taken together these data show that the intracellular domain between TM3 and TM4 of the GABA<sub>C</sub> receptor p1 subunit interacts directly with at least two proteins, namely MAP1B and GLYT-1E/F. It is currently unknown if the interaction of p1 subunit with these two proteins occurs concurrently or sequentially, and if the interactions are cooperative or mutually exclusive. Concurrent interaction between p1, MAP1B, and GLYT-1E/F may serve as a means of targeting or anchoring this transporter to the synapse. Further studies are necessary to determine if the interaction between p1 and GLYT-1E/F influences the functional properties of either protein. For example, it would be of great interest to investigate whether the single-channel characteristics of the GABA<sub>C</sub> receptor are affected by the binding, and possibly even the activity of GLYT-1E/F.

What Possible Role Is There for a Co-localization between GLYT-1E/F and GABA<sub>C</sub> Receptors in Retinal Physiology?—
Retinal bipolar cells receive glutamatergic inputs from photoreceptors at their dendrites, GABA-/glycinergic inputs from amacrine cells at their axon terminal, and deliver glutamatergic output to retinal ganglion cells (34). GABA<sub>C</sub> receptors are expressed predominantly in bipolar cell axon terminals, and colocalization with GLYT-1E/F in this region could effect communication between bipolar and ganglion cells by modulating: 1) the amount of glutamate released from bipolar cells, and 2) the responsiveness of ganglion cell NMDA receptors by controlling the synaptic concentration of glycine, an NMDA receptor agonist (35).

Bipolar cells are non-spiking, i.e., they do not carry action potentials, and the amount of glutamate released from the bipolar cell terminal is graded according to the extent of depolarization. GABA<sub>C</sub> receptors are ligand-gated chloride channels that hyperpolarize the cell when activated, causing a reduction in Ca<sup>2+</sup> influx and a consequent decrease in glutamate release (11, 12). GLYT-1 mediated glycine influx is electrogenic, resulting from the net movement of one positive charge into the cell per glycine molecule, causing the cell to depolarize (36). The magnitude of transporter-mediated depolarization depends upon both the rate of influx and transporter number. GABA<sub>C</sub> receptor and GLYT-1E/F transporter colocalization in bipolar cell axon terminals could thus modulate the membrane potential bi-directionally and regulate glutamate release.

NMDA receptor channel gating requires allosteric glycine binding in addition to glutamate (35). GLYT-1E/F localization to bipolar cell terminals would control the glycine concentration at the synapse. GLYT-1 mediated influx lowers the synaptic concentration of glycine, resulting in a reduction in

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**Fig. 8. Interaction of full-length p1 and full-length GLYT-1E/F in COS cells.** COS cells were co-transfected with full-length GLYT-1E and p1<sup>myc</sup> constructs, and analyzed after 16 h expression. A, co-transfected COS cells were prepared for immunofluorescence and stained with anti-GLYT-1E/F (rhodamine) and anti-Myc 9E10 (fluorescein). The right-hand panel represents the merged image. B, co-transfected COS cells were lysed in 1% Triton X-100 and incubated with 5 µg of anti-GLYT-1E/F (+), or non-immune IgG (−), and precipitated with protein-A-Sepharose. Bound proteins were separated by SDS-PAGE, and the presence of p1 detected by Western blotting with anti-Myc 9E10, in (B) represents 2% of input.
NMDA receptor channel gating (37). In addition, Na⁺-dependent neurotransmitter transporters can function in reverse to release transmitter into synapses (38). Depolarization and raised internal [Na⁺] of the bipolar terminal could result in transporter-mediated glycine release, as well as conventional vesicular release of glutamate. Concurrent release of glutamate and glycine into the synapse would favor NMDA receptor activation. When the bipolar cell terminal hyperpolarizes, glutamate release is diminished and transporter-mediated glycine uptake is favored. This would lower the concentration of glutamate and glycine in the synaptic cleft, reducing NMDA receptor activation. Colocalization of GABA<sub>C</sub> receptors and GLYT-1E/F in bipolar cell axon terminals may play a role in the integration of GABA and glycineric signaling and the modulation of glutamatergic neurotransmission between bipolar cells and retinal ganglion cells.

It should be noted that GLYT-1 does not transport GABA (this study and Ref. 39), and also that homomeric ρ1 GABA<sub>C</sub> receptors are particularly unresponsive to glycine, showing a 10,000-fold lower affinity to glycine compared with GABA, and a very low response to even high doses of glycine (40). However, these authors report a significant potentiating effect of glycine on GABA-activated receptor currents. It is conceivable that colocalization of GLYT-1E/F with ρ1 subunits is important for the regulation of glycine concentrations in the vicinity of the GABAC receptor and thus the fine-tuning of GABAC-mediated responses.

In conclusion, we have isolated a novel GLYT-1 C-terminal variant that interacts with GABA<sub>C</sub> receptors. We show that heterologous expression of variants containing the novel C terminus produce functional glycine transporters pharmacologically identical but kinetically different from known GLYT-1 transporters, and discuss the implications this novel variant has on modeling transporter topology. This is the first report to demonstrate a direct protein-protein interaction between a neurotransmitter receptor and a neurotransmitter transporter.

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