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Cloning, immunolocalization, and functional expression of a GABA transporter from the retina of the skate

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Abstract

Termination of GABA signals within the retina occurs through high-affinity reuptake of the released neurotransmitter by GABA transporters (GATs) present in neurons and glia surrounding the release site. In the present work, we have cloned a novel GAT from the retina of the skate (*Raja erinacea*). The clone codes for a 622 amino acid protein whose sequence has highest similarity to the GABA/β-alanine transporter of the electric ray (*Torpedo marmorata*) (88% identity) and the GAT-3 isolated from rat brain (75% identity). The protein was expressed in *Xenopus* oocytes and characterized using the two-electrode voltage-clamp technique. Application of GABA induced a dose-dependent inward current, with 8 μM GABA producing a half-maximal response. The current required the presence of extracellular sodium and was unaffected by the GABA receptor blocker picrotoxin or the GAT-1 specific antagonist NO-711. The high homology between the cloned skate GABA transporter and the GAT-3 equivalents of other species, coupled with the strikingly similar pharmacological profile to GAT-3s of other species, lead us to conclude that we had cloned the GAT-3 homologue for the skate. Polyclonal antibodies specific to GAT-3 and the previously cloned skate GAT-1 transporter were used to examine the distribution of GAT-3 and GAT-1 immunoreactivity in the retina and in isolated cells of the skate. Antibodies for both transporters showed labeling in the outer and inner plexiform layers, and staining extended from the outer to inner limiting membranes. Both GAT-1 and GAT-3 antibodies labeled enzymatically isolated Müller cells, while bipolar cells and horizontal cells did not appear to express either transporter. These results imply that GAT-1 and GAT-3 are both present in Müller cells of the skate retina where they are likely involved in regulating extracellular concentrations of GABA.

Keywords: *Xenopus* oocyte, Immunocytochemistry, Müller cell

Introduction

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the vertebrate central nervous system. In the vertebrate retina, many interneurons (horizontal cells, amacrine cells, and interplexiform cells) are thought to utilize GABA as their neurotransmitter (cf. Massey & Redburn, 1987 for review). Release of GABA into the outer plexiform layer (OPL) by horizontal cells has been hypothesized to be involved in generation of the surround receptive fields of bipolar cells (Yazulla, 1985; Werblin, 1991; Wu, 1992), and release of GABA in the inner plexiform layer (IPL) by amacrine cells is believed to play a role in directionally selective responses of ganglion cells (Daw & Wyatt, 1976; Caldwell et al., 1978; Ariel & Daw, 1982; Vaney & Young, 1988).

Termination of the GABA signal is mediated predominantly by proteins called GABA transporters (GATs). These proteins are members of a larger family that includes transporters for glycine, serotonin, taurine, and dopamine (Nelson, 1998). All of these transporters have in common a dependence on the sodium and chloride concentration gradients for transport function. Hydropathy analysis suggests that these proteins possess 12 transmembrane regions and that both N- and C-termini reside within the cell (Guastella et al., 1990; Bennett & Kanner, 1997).

Molecular cloning has revealed four types of vertebrate GABA transporters (GAT-1, GAT-2, GAT-3, and the betaine/GABA transporter [BGT-1]; reviewed in Nelson, 1998). A high degree of amino acid homology exists among the members of this class of proteins, especially in the putative transmembrane regions. Despite this, differences in sequence and pharmacologic properties allow them to be separated into their respective classes. For example, GABA uptake by rat GAT-1 and its orthologues cloned from other species can be specifically blocked by several compounds, including tiagabine, NO-711, and SKF 89976-A (Borden et al., 1994*a*). The transport of GABA by rat GAT-2 and rat GAT-3 is not readily blocked by these compounds (Borden et al., 1994*a*). Moreover, these latter two proteins transport the amino acid β-alanine with

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relatively high affinity ($K_m < 110\ \mu$M), while β-alanine is a poor substrate for transport by rat GAT-1 ($K_m > 2000\ \mu$M, reviewed in Palacin et al., 1998). The affinity for GABA of the mouse homolog of the BGT-1 transporter (named mouse GAT2) is approximately 10-fold lower than that of mouse GAT1 ($K_m = 79\ \mu$M *vs.* 6 μM), and this transporter does not show significant uptake of β-alanine (Lopez-Corcuera et al., 1992; Palacin, 1998). The transporters also show substantial differences in sequence in their N- and C-terminal regions. Moreover, while GAT-2 and GAT-3 share many functional properties, they can generally be distinguished by the length of their amino acid sequence. GAT-3 is typically longer than the GAT-1 and GAT-2 sequences by 20–30 amino acids and the human BGT-1 and mouse GAT2 sequences by 8–18 amino acids.

The all-rod retina of the skate (*Raja erinacea/R. ocellata*) has served as a useful model system for examining the role that GABA in general and GABA transport in particular may play in retinal function. GABA immunoreactivity has been observed in cells within the inner nuclear and ganglion cell layers, as well as in horizontal cells of the skate retina (Agardh et al., 1987). Immunolocalization of glutamic acid decarboxylase (GAD), the enzyme responsible for synthesis of GABA from glutamic acid, has been identified in fibers of the inner and outer plexiform layers, cell bodies in the inner nuclear and ganglion cell layers, and in certain horizontal cells (Brunken et al., 1986; Agardh et al., 1987). Early studies using eyecup preparations to examine the uptake of [^{3}H] GABA in skate retina revealed prominent labeling of Müller cells (the radial glia of the retina) with uptake also in presumed amacrine cells (Lam, 1975; Bruun et al., 1984). Electrophysiological and pharmacological studies have confirmed that Müller cells of skate possess transporters for GABA (Qian et al., 1993). Moreover, such studies have also shown that horizontal cells of skate possess a GABA transport mechanism. In voltage-clamped horizontal cells, GABA elicits an inward current with all the hallmarks of a transport-mediated response: the current requires the presence of extracellular sodium and chloride, is blocked by specific GABA transport inhibitors, and is unaffected by $GABA_A$ and $GABA_B$ receptor agonists and antagonists (Malchow & Ripps, 1990; Malchow & Andersen, 2001; Kreitzer et al., 2003). It is not yet known which of the GABA transporter isoforms are expressed by these two cell types, how their functional properties might differ, or whether cells might express multiple isoforms of GABA transporters.

In the present study, we describe our success in cloning a novel GABA transporter from the skate retina and characterizing its response properties when expressed in the *Xenopus* oocyte expression system. This transporter has high homology to GAT-3 transporters cloned from other species and also exhibits a pharmacological profile similar to that observed for other GAT-3 proteins. In addition, we have characterized the distribution of this and another GABA transporter (skate GAT-1) in cells of the skate retina using subtype specific antibodies, and find that both GABA isoforms are clearly expressed in the Müller cells, while horizontal and bipolar cells do not express either transporter. These results further strengthen the contention that Müller cells of skate are likely to play an important role in the regulation of extracellular levels of GABA within the retina.

Materials and methods

Cloning of skate GAT-3 transporter

Skate (*Raja erinacea*) were purchased from the Marine Biological Laboratories (Woods Hole, MA). All experiments conformed to guidelines for animal care established by the Animal Care Committee of the University of Illinois at Chicago. RNA was isolated from skate retina using TRIzol reagent (Invitrogen, Carlsbad, CA.) according to the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed in a volume of 100 μl, which contained 10 μg of RNA and 200 ng each of GAT-specific degenerate primers (5′-TAYTTYTGYATHTGAAGGG and 5′-AANGCCAGNCCVGGDCCTCA). These primers encode for conserved amino acid sequences located in the fourth and fifth transmembrane regions of GABA transporters from mouse (GAT1-4), human (GAT-1), skate (GAT-1), and *Torpedo* (GAT-1). The amplified fragment was cloned into pGEM-T vector (Promega; Madison, WI) and sequenced. The entire coding region of the transporter was isolated using GAT-3-specific degenerate primers (5′-GTGGGATCCAGCGTCATGASWGCGAGMAA and 5′-CGCCGAATTCTCAGAAGTGYGTCTCYTT) based on sequences from *Torpedo* and rat, which include the start and stop codons, respectively. The underlined regions are BamHI (first primer) and EcoRI (second primer) restriction sites used for cloning the fragment into the pCS2+ vector. The clones were sequenced at the University of Chicago Sequencing Center. Each fragment was sequenced in both the forward and reverse direction, and several clones from separate PCR reactions were sequenced on separate occasions to ensure accuracy. An alignment was performed using the ClustalW program (Thompson et al., 1997), and hydropathy analysis was performed using the SOSUI software (Tokyo University of Agriculture & Technology). The SOSUI system analyzes protein sequences, looking specifically at solubility of membrane proteins, location of probable transmembrane regions, presence or absence of a signal peptide, and existence of transmembrane helical regions. This information was used to construct a predicted secondary structure of skate GAT-3. TMPred software was used to generate a hydropathy analysis schematic (Hofmann & Stoffel, 1993). A dendrogram was generated using the TreeView software (Page, 1996) based on the Clustal W alignment program.

Polyclonal GAT antibodies

Anti-GAT-3 antibody was purchased from Calbiochem (San Diego, CA). Antisera specific for skate GAT-1 were generated against a hydrophilic peptide corresponding to skate GAT-$1_{577–598}$ by Biosynthesis, Inc. (Lewisville, TX). The sequence in this region has been shown to be GAT-1 specific in other species and was also distinct when compared to the sequence we obtained of the skate GAT-3 protein. Affinity purification was performed with the peptide using the SulfoLink Coupling Gel Column Kit (Pierce, Rockford, IL). For double-labeling experiments, rabbit polyclonal anti-glutamine synthetase was provided as a kind gift from Dr. Paul Lindser (Whitney Labs, University of Florida, FL). Control experiments to insure specific staining by antibodies included incubation of retinal slices, *Xenopus* oocytes, and CHO cells with primary antibody that had been blocked by preincubation for at least 3 h with a large molar excess (approximately 100-fold) of the corresponding synthesized peptide before incubation of the antibody with the tissue. The GAT-3 antibody was incubated with peptides corresponding to either the C-terminal end of skate or rat GAT-3 (synthesized by Biosynthesis, Inc. Lewisville, TX), while the GAT-1 antibody was incubated with a peptide corresponding to the C-terminal end of skate GAT-1. Images comparing blocked and unblocked antibody labeling were acquired and digitally processed identically.

Western blot

Tissue was collected from skate brain, retina, liver, and muscle and sonicated in sodium dodecyl sulfate (SDS) buffer (1% SDS, 1 mM EDTA at pH 8.0, 10 mM Tris at pH 8.0). Twenty micrograms each of protein sample were mixed with sample buffer (45 mM Tris, 2.5% SDS, 6.5% glycerol, 0.01% bromophenol blue, 3.3% β-mercaptoethanol at pH 8.0) and denatured at 95°C for 5 min. The samples were run on a 7.5% acrylamide gel and transferred to a nitrocellulose membrane. The blot was blocked in Tris-Buffered Saline Tween (TBST) (140 mM NaCl, 20 mM Tris base at pH 8.0, 0.1% Tween) and 3% bovine serum albumin (BSA) for 1 h, reactivated with 60% methanol, and rinsed in TBST. The blot was then incubated with anti-GAT-1 (1:800) in TBST plus 1% BSA at 4°C overnight. This was followed by three sequential 10-min washes in TBST at room temperature. After washing, horseradish peroxidase (HRP)-conjugated secondary antibody (goat-anti rabbit, Fisher, Pittsburgh, PA, 1:5000) was applied in TBST plus 1% BSA for 1 h at room temperature. The signal was detected with the SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL) per the manufacturer's protocol.

Immunohistochemistry

Skate retinas were fixed for 2 h in cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and cryoprotected at 4°C by stepping through cold 20% (8 h) and 30% sucrose overnight. The tissue was then mounted with O.C.T. (Miles, Inc., Pittsburgh, PA), sectioned at 20 μm, and picked up on Superfrost Plus Slides (Fisher Scientific, Pittsburgh, PA). The slides were allowed to dry at room temperature for 2 h and were stored at −20°C. Solitary retinal cells were dissociated according to the procedure described in Malchow et al. (1989). Cells were plated on poly-L-lysine coated coverslips (Fisher Scientific, Pittsburgh, PA) in culture dishes and allowed to adhere for 30 min at room temperature. Adherent cells were fixed for 40 min in 4% paraformaldehyde in skate Ringer (250 mM NaCl, 6 mM KCl, 20 mM $NaHCO_3$, 1 mM $MgCl_2$, 4 mM $CaCl_2$, 360 mM urea, 10 mM glucose, and 10 mM HEPES, pH 7.6) and thoroughly washed in 0.1 M sodium phosphate buffer, pH 7.4.

Samples of skate retina or isolated cells were blocked for 2 h (4% goat serum, 2% bovine gamma globulin, and 0.3% Triton X-100 in PBS, pH 7.6) and incubated in primary antisera overnight at room temperature (1:800 for GAT-1 and 1:100 for GAT-3 in blocking solution). The sections were washed for 10 min in 0.1 M sodium phosphate buffer (pH 7.6) three times before a 2-h incubation in Cy-3 tagged goat anti-rabbit secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:800 in blocking solution. The sections were washed three times for 10 min in 0.1 M sodium phosphate buffer, pH 7.6. After washing, the sections were coverslipped with Vectashield (Vector Laboratories, Burlingame, CA) and analyzed using a Zeiss confocal microscope. For double labeling of isolated cells, the secondary antibody for GAT-1 and GAT-3 was Cy3-tagged goat anti-rabbit antibody with Fab fragments (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:50 in blocking solution and incubated for 2 h. Glutamine synthetase rabbit antiserum was diluted 1:100 in blocking solution and then applied to the tissue overnight. This was followed by a 2-h incubation in goat anti-rabbit secondary antibody tagged with a fluorescein isothiocyanate (FITC) fluorophore, which was diluted 1:100 in blocking solution. Four washes (10-min each) were performed in 0.1 M sodium phosphate buffer, pH 7.6, between each step. The cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and analyzed using a Zeiss confocal microscope. The glutamine synthetase antibody was tested on isolated retinal cells, and it recognized only epitopes present in the skate Müller cells, as reported by Linser et al. (1984). In double-labeling experiments using both GAT antibodies and antibodies to glutamine synthetase, control experiments were performed in which the glutamine synthetase antibody was omitted to ensure that the GAT antibodies bound in the sample were saturated by the first secondary antibody under our experimental conditions. Under this control condition, the FITC-tagged secondary antibody did not label Müller cells or any other cells in the dish.

Expression of skate GATs in Xenopus oocytes

Capped RNA was transcribed using the mMessage mMachine High Yield Capped RNA Transcription kit (Ambion, Austin, TX) according to the manufacturer's protocol. Then RNA was aliquoted and frozen at −80°C for up to 6 months. Female *Xenopus* were anesthetized in 0.2% Tricaine (MS-222) (Sigma, St. Louis, MO) for 10 min. Oocytes were surgically removed from the animals and then defolliculated in collagenase Type I (Sigma, St. Louis, MO, 1 mg/ml) for approximately 2 h. Selected oocytes were then incubated in oocyte Ringer's solution (100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 10 mM glucose, pH 7.4) for at least 2 h to recover from enzymatic treatment. Each oocyte was injected with 50 nl of cRNA (0.2–0.6 μg/μl). The cells were cultured in Ringer's solution for 3 days at 19°C before recordings were performed.

Dual-electrode voltage-clamp recordings were obtained using a Geneclamp Amplifier (Axon Instruments, Union City, CA). The amplifier was connected to an Axon Instruments Digidata 1200A analog-to-digital converter. Data were acquired and analyzed using pCLAMP 6.0 software run on an IBM-compatible computer. Recording pipettes were prepared using a Sutter P97 pipette puller (Sutter Instrument, Novato, CA). Electrodes were filled with 3 M KCl and had resistances between 0.5 and 1.5 MΩ. Currents for each cell were normalized to the maximum response and the average of the normalized data at each concentration was used in the dose–response curves that were constructed using Origin 6.0 (OriginLab Corporation, Northampton, MA). Data were represented as mean ± SEM.

Results

Cloning of a skate GAT-3 transporter

A GABA transporter sequence of 1869 basepairs with an open reading frame of 622 amino acids was cloned from the skate retina using degenerate primers to conserved regions of known GABA transporters. A schematic of the predicted amino acid sequence is shown in Fig. 1A. Consistent with previous models for the topology of GABA transporters, the protein is predicted to span the membrane 12 times and to have intracellularly residing termini, reflecting the apparent absence of a signal peptide in the N-terminal end of the protein (Guastella et al., 1990). The locations of the transmembrane regions were predicted through hydropathy analysis (Hofmann & Stoffel, 1993), shown in Fig. 1B, with each peak representing a probable transmembrane region. The two most likely topologies, which overlap significantly, are shown. The darker line represents the most likely prediction, and this was used to generate the secondary structure schematic in Fig. 1A.

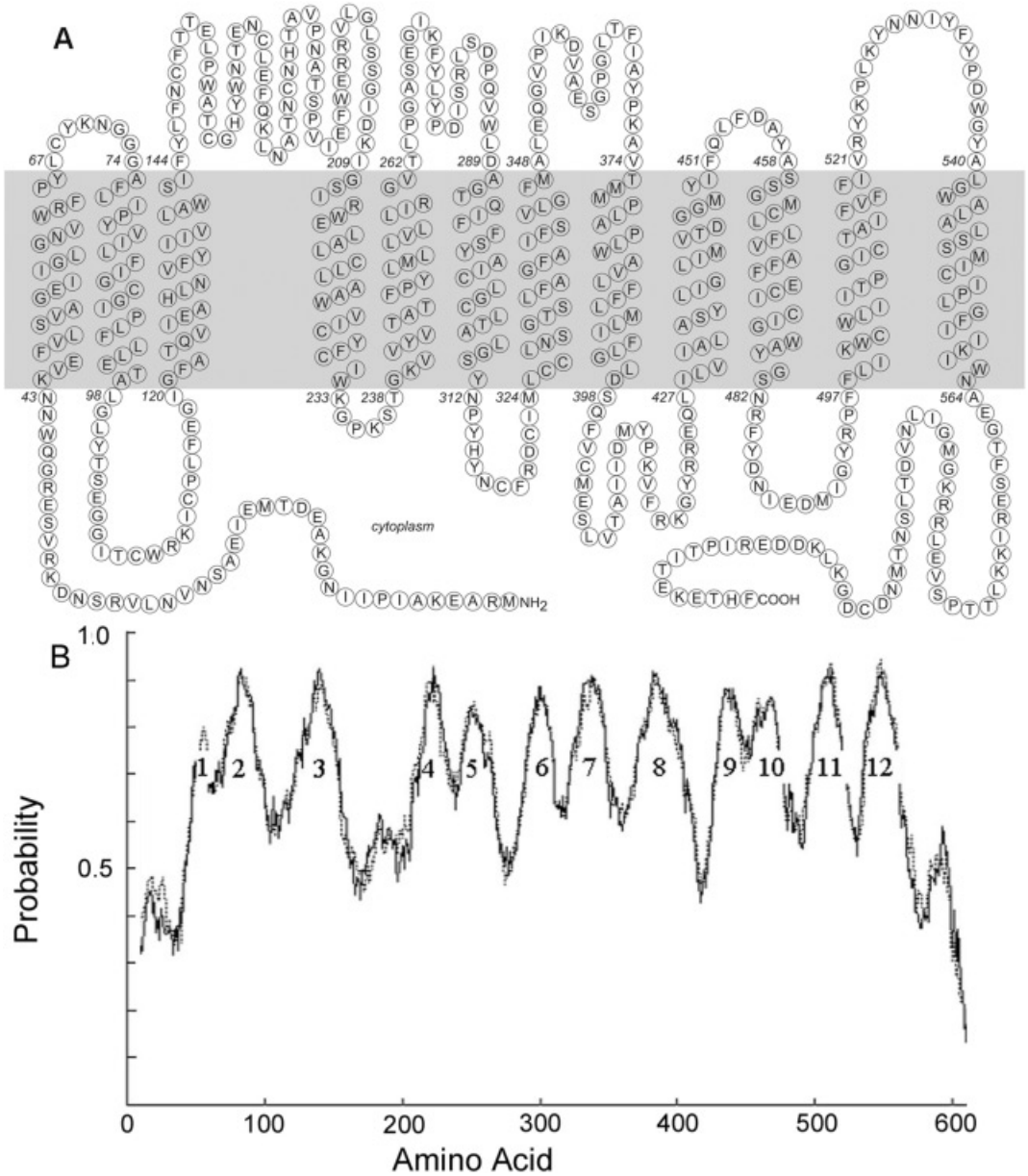


Fig. 1. Predicted amino acid sequence of skate GAT-3. A: The schematic of skate GAT-3 showing the predicted 12 transmembrane topology based on hydropathy analysis. Each amino acid directly adjacent to a transmembrane region is numbered. Note the PDZ binding motif (KETHF) at the carboxyl terminus of the protein. B: Hydropathy analysis of skate GAT-3. Each peak represents a probable transmembrane region. The TMPred program predicted two possible topologies, which overlap significantly. The darker line represents the most likely prediction and was used to generate the schematic in A. The less likely of the two topologies is represented by the lighter intensity (dotted) line, which is most visibly different in the region preceding the first transmembrane region. *X* axis—amino acid number; *Y* axis—transmembrane probability.

To investigate the relationship of skate GAT-3 with other GATs, a dendrogram was generated to show the degree of homology between the skate GAT-3 and GATs from other species. The information for the tree was acquired through the ClustalW alignment of amino acid sequences for skate GAT-3 with other known GAT sequences available at the National Center for Biotechnology Information (NCBI). As shown in Fig. 2, the skate GAT-3 transporter shows the highest similarity to the *Torpedo* GABA/β-alanine transporter (Guimbal et al., 1995) and high similarity to rat and human GAT-3 (Borden et al., 1992, 1994*b*) and the mouse GAT4 (Liu et al., 1993). Note that, due to historical naming conventions, the rat and human GAT-3 proteins are homologous with mouse GAT4, rat and human GAT-2 are homologous to mouse GAT3, and the BGT-1 transporters from rat and human are homologous to mouse GAT2. Skate GAT-3 is 88% identical to the *Torpedo* GABA/β-alanine transporter in both nucleotide and amino acid sequence. The rat, mouse, and human orthologues are 68% identical to skate GAT-3 in nucleotide sequence within the coding region and 75–76% identical in amino acid sequence.

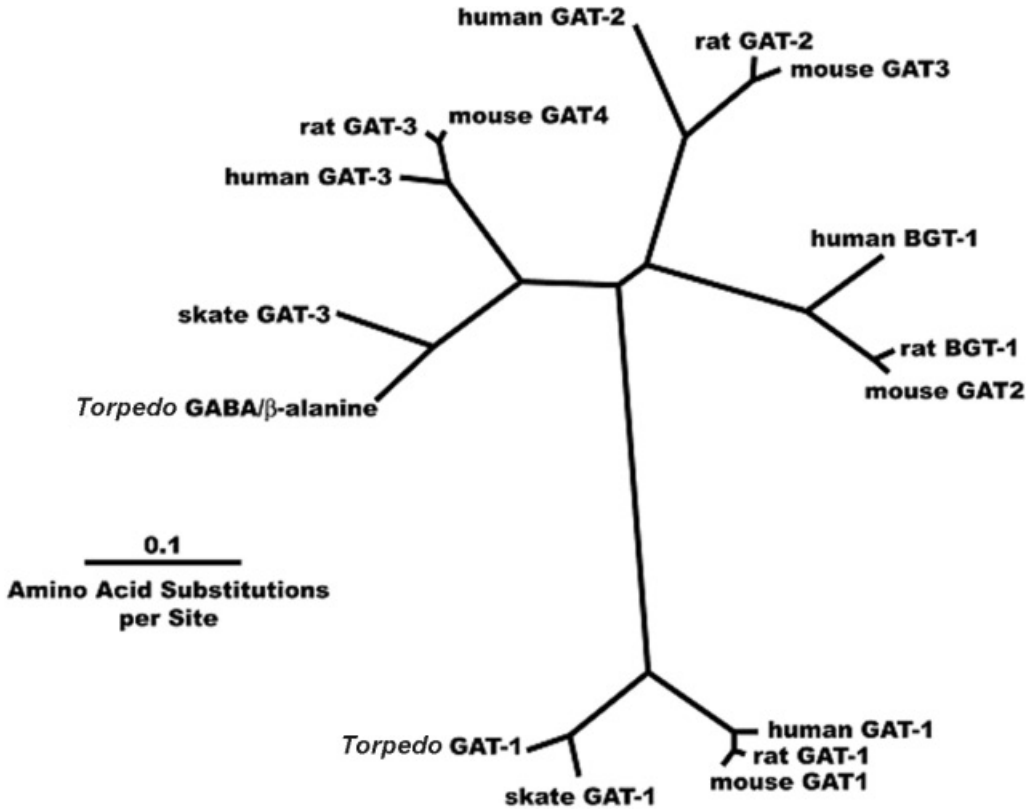


Fig. 2. Evolutionary analysis of vertebrate GABA transporters. Amino acid sequences for known vertebrate GABA transporters were aligned using the Clustal W program. The results are depicted in an unrooted tree using the TreeView program. The skate GAT-3 sequence is most similar to the GABA/β-alanine GABA transporter of *Torpedo*. Note differences in naming terminology: the mouse homologue labeled GAT2 is actually most closely related to the BGT-1 transporters, the mouse GAT3 is most homologous to the transporters typically labeled as GAT-2, and the mouse GAT4 is most closely related to GAT-3s of other species.

Expression of skate GATs in Xenopus oocytes

Functional properties of the skate GAT-3 protein were determined using the *Xenopus* oocyte expression system and two-electrode voltage-clamp recording techniques. In oocytes expressing skate GAT-3, the application of 100 μM GABA produced inward currents of several tens of nanoamps in magnitude that were maintained for as long as the GABA was applied (Fig. 3A). The GABA-elicited current was dependent upon the presence of extracellular sodium. Replacing all the extracellular sodium in the bath with a comparable amount of N-methyl-D-glucamine (NMDG) completely abolished the inward current elicited by GABA. The effect was reversible, as returning the oocyte to extracellular solution that contained sodium produced a response that was similar to the first trace. In the absence of sodium, the current was $0.8 \pm 0.06\%$ ($n = 4$) of the amplitude recorded in the same cells in the presence of sodium.

The current–voltage relationship of the currents elicited by GABA is shown in Fig. 3B. In these experiments, the voltage was ramped from -100 mV to 50 mV at 0.5 mV/ms in the presence of GABA (100 μM) three times, and the results averaged. The same voltage ramp protocol was then applied in the absence of GABA. The plot in Fig. 3B represents the subtraction of currents in Ringer's solution without GABA from currents obtained in the presence of GABA. GABA-evoked currents in GAT-3-expressing oocytes were highly voltage dependent. The GABA-elicited current was largest at the most negative voltages and became progressively smaller as the cell was depolarized. Significantly, the current never reversed into an outward current even at voltages of $+50$ mV. The current–voltage relation we have obtained for the skate GAT-3 protein is thus very similar to the current–voltage relations for GABA transporters obtained in oocyte expression studies for GAT-1 (Guastella et al., 1990; Ruiz et al., 1994) and for GABA transporters normally expressed in a variety of cell types (Guimbal et al., 1995; Risso et al., 1996; Malchow & Andersen, 2001).

The GABA dose–response curve for skate GAT-3 is shown in Fig. 3C. In these experiments, cells were voltage clamped at -70 mV, and the peak current amplitude at each concentration was recorded and normalized to the maximum response obtained with 1 mM GABA. The data were fit to the Hill equation to produce dose–response relationships. The dose of GABA producing a half-maximal response (EC_{50}) was 7.7 ± 1.3 μM from 12 cells. The Hill coefficient (n) was calculated to be 0.85, suggesting that one molecule of GABA was likely transported for each cycle of activation of the transporter. We also examined the response to β-alanine and found that this naturally-occurring amino acid also induced an inward current in the oocytes. The dose–response relationship for β-alanine measured from seven cells had an EC_{50} of 105.6 ± 9.5 μM and a Hill coefficient of 0.93. The maximal response produced by β-alanine was only 73% of the maximal GABA response. Finally, we examined the effects of nipecotic

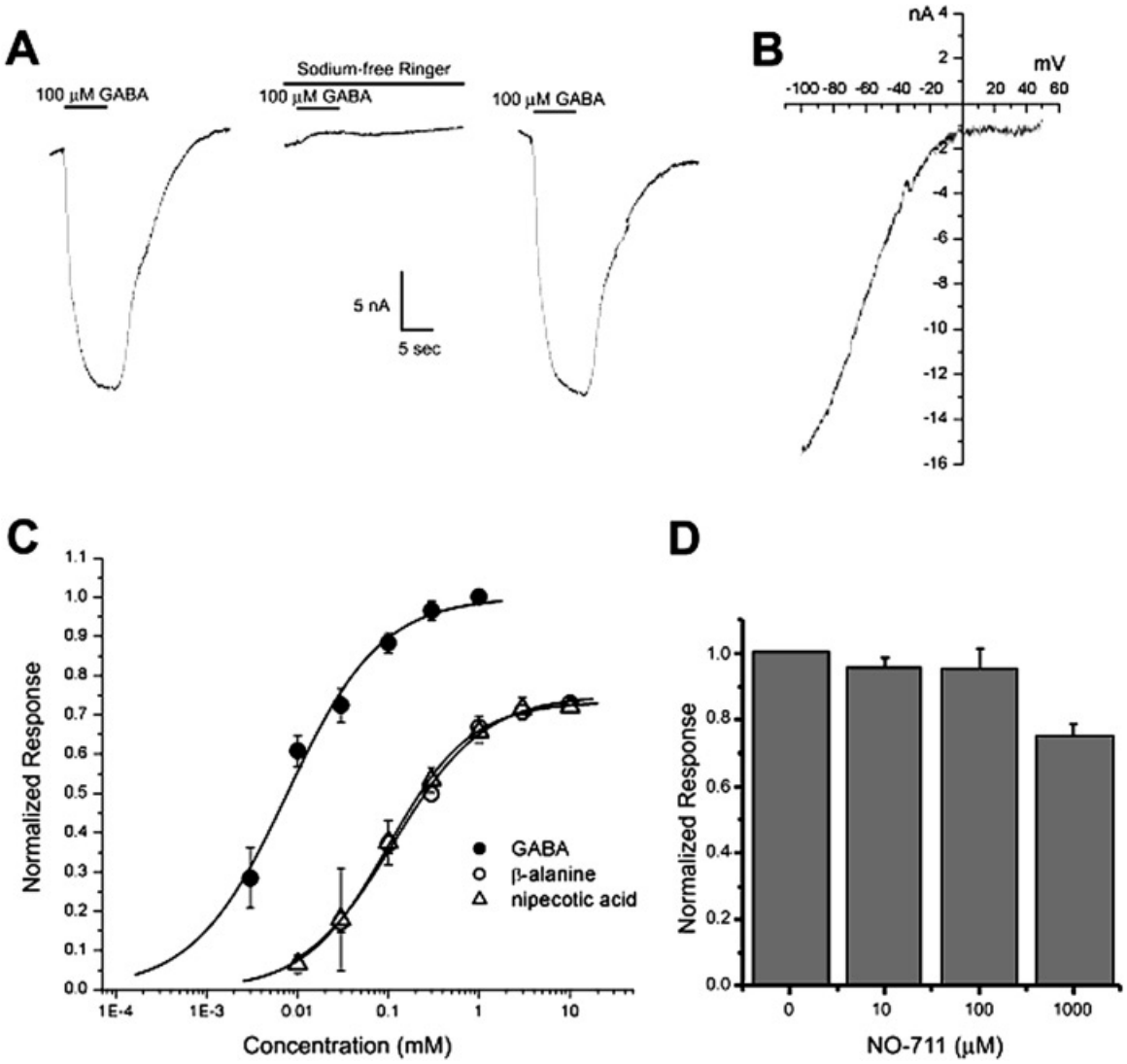


Fig. 3. Expression of skate GAT-3 in *Xenopus* oocytes. A: An inward current is produced when 100 μM GABA is applied to an oocyte expressing the skate GAT-3 protein. Replacing the sodium in the extracellular solution with NMDG abolished the GABA current in the same oocyte. The GABA-induced current returned after the oocyte was placed back in Ringer's solution containing the normal amount of extracellular sodium. B: A ramp protocol was used to determine the *I–V* relationship of the GABA-elicited current. The plot was produced by subtracting the mean currents measured in oocyte Ringer's solution from those measured in the presence of 100 μM GABA. C: The substrates GABA, β-alanine, and nipecotic acid all induced inward currents in oocytes expressing the skate GAT-3 protein. The continuous curves were fit to a Hill equation with EC_{50} of 7.7 ± 1.3 μM for GABA, 105.6 ± 9.5 μM for β-alanine, and 90.1 ± 8.5 μM for nipecotic acid. Data were collected from 12 (GABA), 7 (β-alanine), and 9 cells (nipecotic acid). Error bars represent standard error. D: Three concentrations of NO-711 were applied to oocytes expressing skate GAT-3 in the presence of 100 μM GABA. The current was reduced by 4.6 ± 0.03% in 10 μM NO-711, 5.4 ± 0.06% in 100 μM NO-711, and 25 ± 0.04% in 1 mM NO-711.

acid, reported to act as a competitive inhibitor of certain GABA transporters (Liu et al., 1993). Nipecotic acid by itself also induced an inward current; in nine cells expressing GAT-3, nipecotic acid produced a maximal inward current that was 72% as large as that produced by GABA. The EC_{50} for the nipecotic acid-induced current was 90.1 ± 8.5 μM, and the curve was best fit with a Hill coefficient of 0.9.

GAT-3 transporters differ from GAT-1 transporters in their sensitivity to the noncompetitive GABA transport blocker NO-711, with GAT-1 transporters generally showing considerable sensitivity to NO-711, while GAT-3 transporters demonstrate relatively little inhibition (Borden et al., 1994*a*). As shown in Fig. 3D, 10 μM and 100 μM NO-711 had very little effect on responses elicited by 100 μM GABA; the GABA-induced current was reduced by only 4.6 ± 0.03% in 10 μM NO-711 and 5.4 ± 0.06% in 100 μM NO-711. Even at the very high concentration of 1 mM, NO-711 produced only a 25 ± 0.04% reduction in the magnitude of the GABA-elicited currents in oocytes expressing skate GAT-3.

In separate experiments, we also expressed in *Xenopus* oocytes the skate GAT-1 protein that had previously been cloned (Qian et al., 1998). Application of GABA elicited an inward current in oocytes expressing the skate GAT-1 protein, and the GABA-

elicited current was completely abolished when all extracellular sodium was replaced by N-methyl-D glucamine, as expected for a sodium-chloride-GABA co-transport protein. The current elicited by 1 mM GABA was markedly reduced by the GABA transport inhibitor SKF-100330-A, with the concentration of SKF-100330-A producing a half-maximal block calculated to be 4 μM ($N = 8$). The GABA dose–response relation of the skate GAT-1 protein was characterized by a surprisingly high value for the concentration producing a half-maximal response—the EC_{50} for the dose–response relation was determined to be 710 ± 140 μM ($N = 15$) with a Hill coefficient of 0.64 ± 0.07. The GABA-elicited current was reduced by NO-711: 10 μM of this GAT-1 inhibitor decreased the current elicited by 1 mM GABA by 12 ± 4%, 100 μM produced a 36 ± 12% reduction, and 1000 μM NO-711 reduced the response by 77 ± 13%. However, concentrations of NO-711 ranging from 0.01 μM to 1 μM did not reduce the GABA-elicited current, and we calculate an IC_{50} value of about 350 μM NO-711 to reduce the GABA-elicited current by half, a value significantly higher than that reported to block other GAT-1 transporters (Palacin et al., 1998). We do not presently understand the reason for the low affinity of the skate GAT-1 to either GABA or NO-711 when expressed in the *Xenopus* oocyte system.

Localization of GAT-1 and GAT-3 in the skate retina

We next used immunolocalization techniques to examine the distribution of skate GAT-1 and skate GAT-3 in the skate retina. A commercially available GAT-3 polyclonal antibody that had been generated against the C-terminal end of the rat GAT-3 protein specifically recognized epitope(s) present in *Xenopus* oocytes expressing the skate GAT-3 protein (Fig. 4D). The antibody did not label oocytes injected with GAT-1 mRNA (Fig. 4C). Positive labeling was blocked when the antibody was preadsorbed with a peptide identical to the C-terminal end of either the rat sequence or the skate sequence, and the antibody did not label native oocytes. The observation that the rat GAT-3 antibody specifically labeled the skate GAT-3 protein is interesting given that the 21 amino acids at the C-termini of the skate and rat GAT-3 proteins share only 62% identity. It seems likely, therefore, that the eight terminal amino acids, which are identical in these species, is the location for the epitope(s) recognized by this antibody.

Commercially available antibodies prepared against a peptide corresponding to the C-terminus of rat GAT-1 failed to display any positive, specific labeling in the skate retina. Consequently, we generated a skate-specific GAT-1 antibody against a peptide identical in sequence to the last 21 amino acids of the skate GAT-1 sequence (Qian et al., 1998). Western blot analysis was performed using the GAT-1 specific antibody against protein extracts from skate brain, retina, liver, and muscle. A band of approximately 60 kDa was recognized in protein from brain and retina, but no labeling was seen in liver or muscle (Fig. 4E). Preadsorbing the antibody with an excess of peptide blocked the specific labeling of this band. The predicted molecular weight of the GAT-1 protein is approximately 70 kDa. The skate GAT-1 antibody was applied to oocytes that expressed either the skate GAT-1 protein or the skate GAT-3 protein. Fig. 4A shows that prominent labeling was observed in oocytes expressing the skate GAT-1 protein. Preadsorbing the antibody with an excess amount of peptide blocked this recognition. The skate GAT-1 antibody did not recognize epitopes within the oocytes expressing the skate GAT-3 protein (Fig. 4B) or native oocytes.

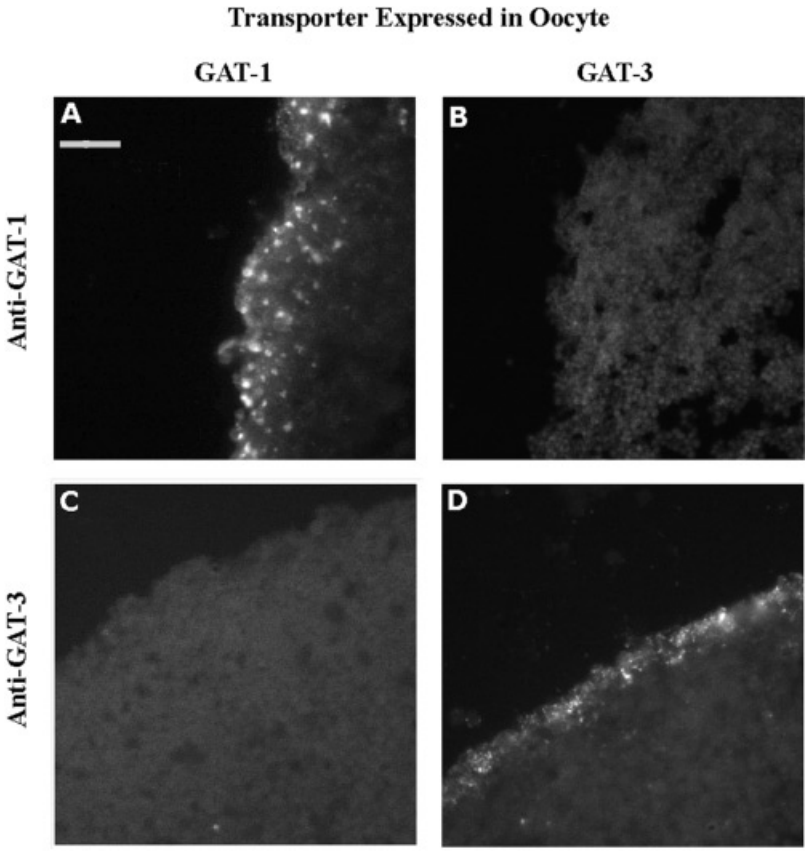
The newly developed skate GAT-1 antibody was applied to skate retinal slices. In Fig. 5A, the GAT-1 antibody displayed prominent labeling in the inner plexiform layer and was also present in the outer plexiform layer. Moreover, positive staining reached as far as the outer limiting membrane. Fig. 5C shows the pattern of GAT-3 labeling using the commercially available antibody developed against the rat GAT-3 C-terminus. Positive labeling was observed in both the inner and outer plexiform layers of the skate retina, and it also reached to and included the outer limiting membrane.

We also examined antibody labeling of isolated retinal cells. Four morphologically distinct skate retinal cells could be readily identified in the culture dish: photoreceptors, Müller cells, horizontal cells, and bipolar cells. Of these cells, only Müller cells showed positive labeling with the GAT antibodies. Fig. 6A shows GAT-1-antibody labeling of a Müller cell. Box 1 is a phase-contrast photomicrograph of an isolated Müller cell demonstrating its unique morphology. This cell has its apical (distal) end towards the left of the photomicrograph. The same cell labeled with the skate GAT-1 antibody, using Cy3-labeled secondary antibody, is shown in box 3. Labeling is seen throughout the entire cell. The same cell was also double labeled with an antibody against the Müller cell marker glutamine synthetase with a FITC-tagged secondary antibody, shown in green in box 2. In control experiments in which the glutamine synthetase antibody was omitted, the anti-GABA transporter antibodies showed the same pattern of labeling as seen in Figs. 6 and 7, but no discernable binding of the FITC-tagged secondary antibody was detected. Over 200 Müller cells were labeled with the GAT-1 antibody, and all showed a similar labeling pattern. The overlay of the two images is shown in box 4, with the yellow color representing the overlap of localization of the two antibodies within Müller cells. The GAT-1 antibody did not recognize epitopes within horizontal cells, nor was positive staining ever observed in bipolar cells or photoreceptors. Fig. 6B shows the typical lack of staining seen in a horizontal cell (phase contrast image in box 1, fluorescent image of same cell in box 3) and in a bipolar cell (phase contrast image in box 2, and fluorescent image of same cell in box 4). Over 100 additional cells of each type were examined. Of note, internal and external horizontal cells are easily distinguished from one another in an isolated cell preparation (Malchow et al., 1990). Neither of the two types of horizontal cells showed evidence of labeling with the GAT-1 antibody.

Fig. 7 shows the results of applying the commercial GAT-3 antibody to isolated retinal cells. Müller cells again were prominently labeled with the GAT-3 antibody (Fig. 7A). An example of a GAT-3-labeled Müller cell is shown in box 3, and the light photomicrograph of this same cell is shown in box 1. The identity of the cells as Müller cells was again confirmed using the antibody against glutamine synthetase (box 2). The overlay of the images in boxes 2 and 3 is shown in box 4, with the yellow color marking areas of colocalization of the two antibodies, and demonstrates extensive labeling of both glutamate synthetase and the GAT-3 antibody along the entire length of the cell. Fig. 7B shows that neither horizontal cells nor bipolar cells exhibited positive staining for GAT-3. Box 1 shows a light photomicrograph of a horizontal cell; the fluorescent image for this same cell showing the lack of labeling is presented in box 3. A similar lack of labeling for GAT-3 is shown for a bipolar cell (boxes 2 & 4).

Discussion

In this work, we have cloned and characterized a novel GABA transporter (GAT) from the retina of the skate. This transporter has



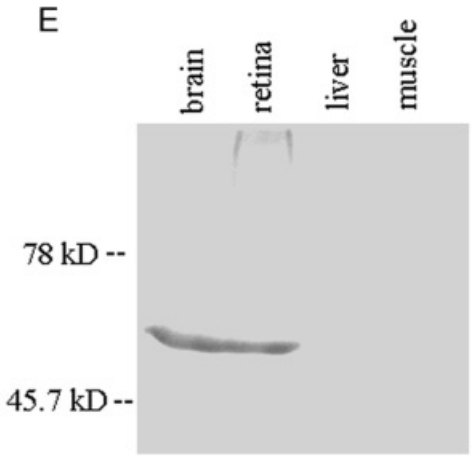


Fig. 4. Immunoreactivity of GAT-1 and GAT-3 in *Xenopus* oocytes. A: Our newly developed GAT-1 antibody recognized epitopes present in oocytes injected with mRNA for skate GAT-1. B: Oocytes injected with mRNA for skate GAT-3 were not labeled by the antibody developed to GAT-1. C: A commercially available GAT-3 antibody did not recognize cells expressing skate GAT-1. D: Positive labeling of oocytes expressing the GAT-3 protein was detected using the antibody to GAT-3. E: Immunoblot using skate GAT-1 polyclonal antibody strongly labeled one band in brain and retina at approximately 60 kDa. No signal was observed in liver or muscle. Preadsorbed GAT-1 did not label the control blot (not shown). Scale bar = 20 μm.

highest similarity with the GABA/β-alanine transporter isolated from the electric lobe of *Torpedo* and the GAT-3 isolated from rat brain. The length of the skate protein is consistent with other GAT-3 proteins, which are between 622–632 amino acids in length. Hydropathy analysis of the skate protein predicts 12 transmembrane regions, with both the N- and C-termini located intracellularly, a topology similar to that predicted for other GATs based on hydropathy analysis (Guastella et al., 1990; Nelson et al., 1990; Borden et al., 1992; Guimbal et al., 1995; Qian et al., 1998). Based on *N*-glycosylation scanning mutagenesis experiments of the rat GAT-1 transporter, Bennett and Kanner (1997) have proposed a revised topology for GABA transporter proteins. In their model, there are still 12 transmembrane segments, but the first putative transmembrane region does not cross the membrane, and instead forms a reentrant loop, and in which the first internal loop is now external. It is possible that the detailed topology of our skate GAT-3 protein could fit into this newer scheme. The loops are thought to be involved in ion permeation when present in ion channels (reviewed in MacKinnon, 1995), but their possible role in transport is not understood. Amino acids in the N-terminal third of GAT proteins have been suggested to play a crucial role in the transport of GABA and may be involved in the binding of sodium and chloride. In one study, conserved charged amino acids located within the putative transmembrane domains of rat GAT-1 were mutated using site-directed mutagenesis. One site, arginine 69 (R^{69}), was shown to be essential for transport activity (Pantanowitz

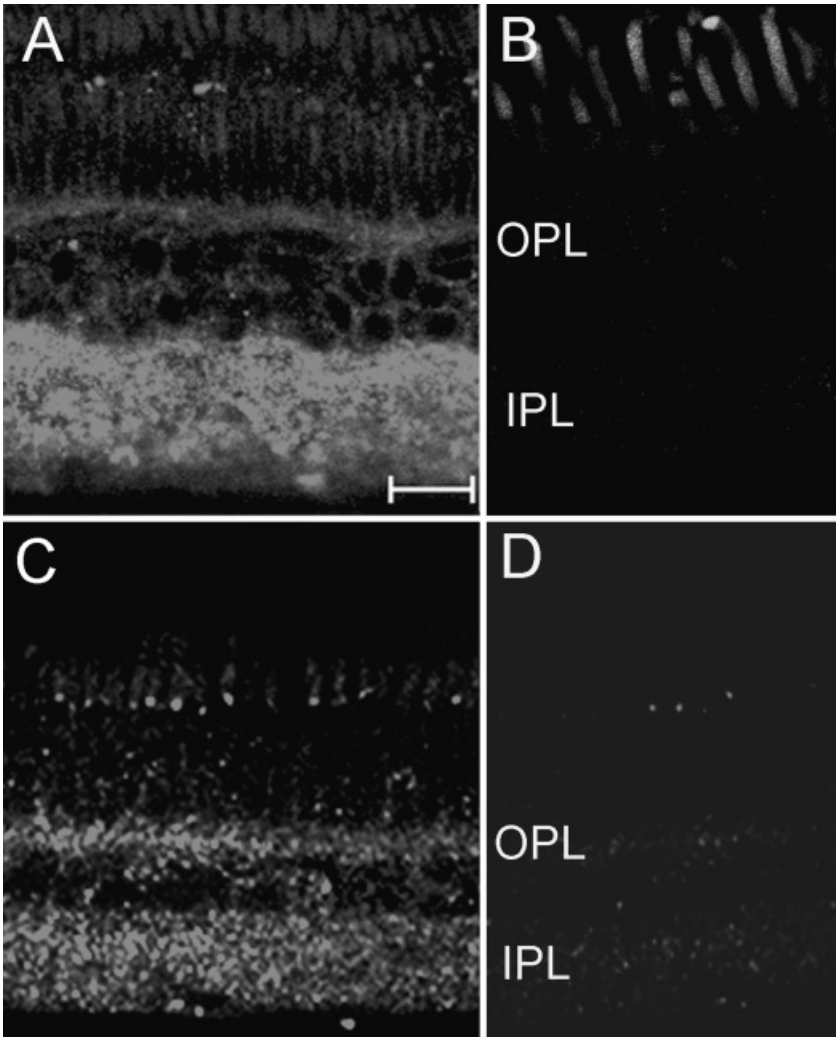


Fig. 5. Immunolocalization in retinal slices using GAT-1 and GAT-3 antibodies. A: GAT-1 antibody labeled the OPL and IPL, as well as processes surrounding cell bodies within the INL. B: GAT-1 antibody labeling was blocked by preadsorption with an excess of peptide. C: GAT-3 antibody recognized processes within both plexiform layers. Some immunoreactivity was also observed in the INL. D: GAT-3 antibody labeling was blocked by preadsorption with an excess of peptide. Scale bar = 20 μm.

et al., 1993). This corresponds to R^{63} in skate GAT-3, a site that is conserved in all known GAT-3 proteins. In rat GAT-1, R^{44} and tryptophan 47 (W^{47}) have also been shown to be important for normal transport function and are thought to be involved with the reorientation of the transporter after release of GABA, sodium and chloride into the cell (Bennett et al., 2000). These amino acids, which correspond to R^{38} and W^{41} in skate GAT-3, are also conserved throughout the vertebrate GABA transporter family. Finally, the highly conserved glutamic acid 101 (E^{101}) of GAT-1 (corresponding to E^{95} in skate GAT-3) was shown to be necessary for at least one of the conformational changes of the rat GAT-1 transport cycle (Keshet et al., 1995). Most of the sequence variability in the GATs of differing species occurs in the termini of the proteins. These highly variable regions are not thought to be necessary for transport function, but may instead be involved in targeting, localization, and modulation of the transporters (Mabjeesh & Kanner, 1992). The variability in these regions indicates that the regulation of these proteins is likely to be different in the four classes of GAT and between species. One example of this specificity is the Post-synaptic density-95/discs large/zonula occludens-1 (PDZ) binding motif at the carboxyl end of skate GAT-3, which is identical to that of human and rat GAT-3. This PDZ binding motif is critical for the difference in subcellular localization of GAT-2 and GAT-3 in epithelial cells (Muth et al., 1998).

When expressed in *Xenopus* oocytes, skate GAT-3 formed a functional GABA transporter on the cell membrane. Application of GABA resulted in an inward current that required the presence of extracellular sodium and displayed significant inward rectification. The pharmacological properties of the expressed skate GAT-3 were very similar to those of other GAT-3 proteins. The skate GABA transporter we have cloned exhibited a high affinity for GABA with 7.7 ± 1.3 μM GABA inducing a half-maximal response. This is similar to that for the *Torpedo* GABA/β-alanine (EC_{50} = 18.7 μM) and rat GAT-3 (EC_{50} = 12 μM) proteins expressed in COS cells (Borden et al., 1992; Guimbal et al., 1995). β-alanine also induced an inward current in oocytes expressing the cloned GAT-3 protein at relatively low concentrations (half-maximal response produced by 105.6 ± 9.5 μM). This value is similar to that reported for *Torpedo* GAT-3 (48 μM; Guimbal et al., 1995). In contrast, GAT-1 transporters do not readily transport β-alanine; the EC_{50} values are typically greater than 2000 μM (reviewed in Palacin et al., 1998). The transport activity mediated by the skate GABA transporter we have cloned was only poorly inhibited by NO-711, even at concentrations of 1 mM. The pharmacological similarity of our skate GABA transporter to GAT-3s of other species, coupled with the high homology between our cloned transporter and the GAT-3 equivalents of other species, leads us to conclude that we have cloned the GAT-3 homologue for

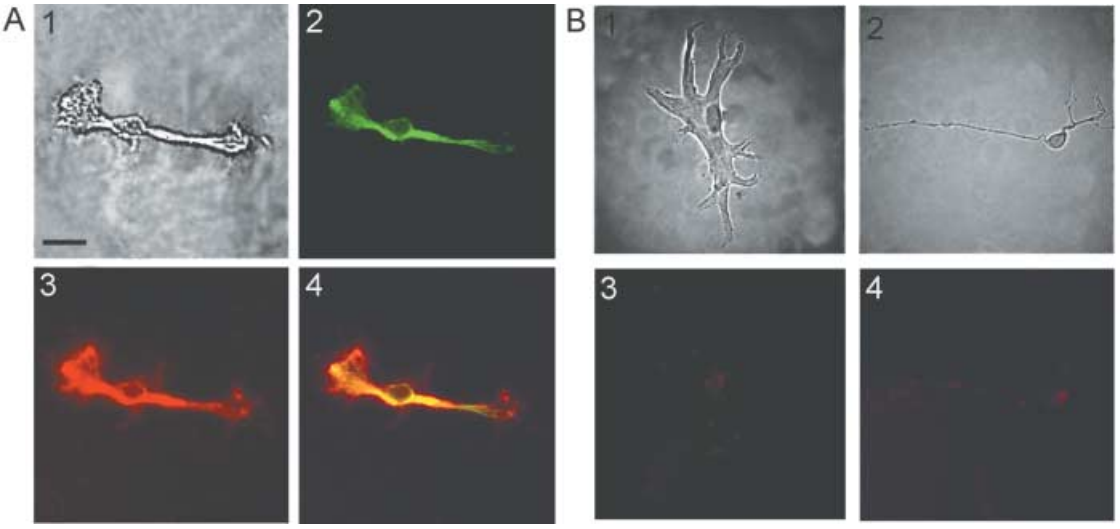


Fig. 6. GAT-1 labeling of isolated retinal cells. A: A Müller cell [light photomicrograph image, 1] displayed positive staining using an antibody for glutamine synthetase (2) and for the antibody directed against skate GAT-1 (3). Colocalization of the two antibodies appears yellow (4). B: light photomicrograph images of a horizontal cell (1) and a bipolar cell (2). Neither the horizontal cell (3) nor the bipolar cell (4) showed positive immunoreactivity for GAT-1 or glutamine synthetase. Scale bar = 10 μm.

the skate. For these reasons, we have chosen to designate the sequence we have cloned as skate GAT-3.

Expression in oocytes of the skate GAT-1 protein previously cloned by Qian et al. (1998) also resulted in GABA-elicited currents that required the presence of extracellular sodium and were blocked by the GABA transport blocker SKF-100330-A. However, the dose of GABA required to produce a half-maximal response was surprisingly high: $\sim$ 710 μM, compared to 2 μM for the GAT-1 cloned from *Torpedo* and 7–12 μM for the rat GAT-1 (reviewed in Palacin et al., 1998). The current could be blocked by NO-711, but required 350 μM of this compound to produce a half-maximal block, a concentration significantly greater than the submicromolar values required to block GAT-1 activity in other species (Palacin et al., 1998). The apparent low affinity for the skate GAT-1 protein to both GABA and NO-711 cannot be accounted for by aberrant point mutations occurring in the clone originally reported by Qian et al. (1998); we completely resequenced their cloned skate GAT-1 transporter and found no difference from the originally reported sequence. It is possible that the disparities in affinity for GABA of the skate GAT-3 and GAT-1 represent true fundamental differences between the proteins, suggesting distinct functional roles in regulating the concentration of

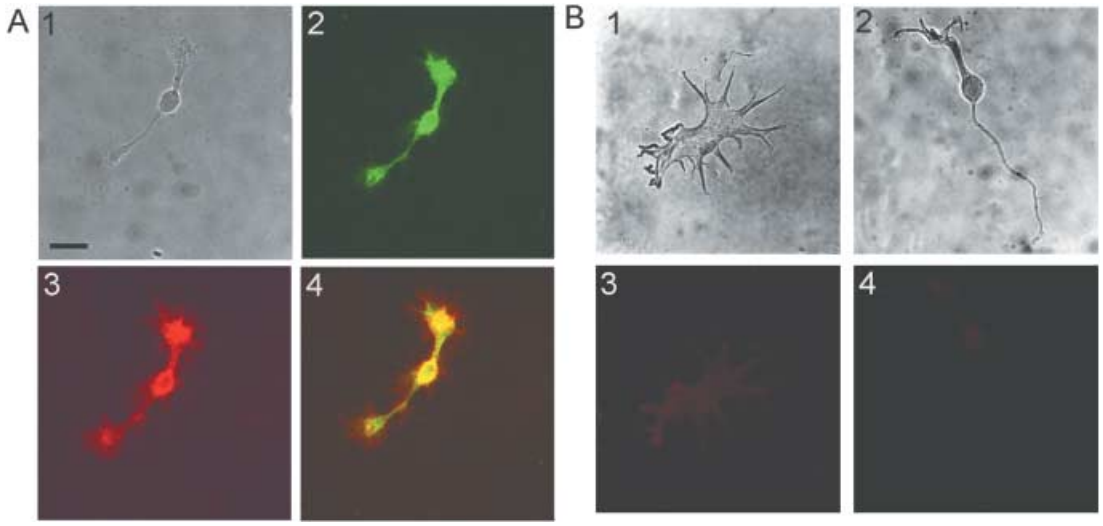


Fig. 7. GAT-3 labeling of isolated retinal cells. A: A Müller cell [light photomicrograph image, 1] displayed positive staining using an antibody for glutamine synthetase (2) and an antibody directed against GAT-3 (3). Colocalization of the two antibodies appears yellow (4). B: light photomicrograph images of a horizontal cell (1) and a bipolar cell (2). Neither the horizontal cell (3) nor the bipolar cell (4) showed positive immunoreactivity for GAT-3 or glutamine synthetase. Scale bar = 10 μm.

GABA in the retina of the skate. The higher affinity of the GAT-3 transporter might enable Müller cells to regulate GABA levels when extracellular concentrations are low, while the lower affinity of the GAT-1 transporter for GABA would make it ideal for regulating higher extracellular concentrations of GABA. While an attractive hypothesis, the marked differences between the properties of the skate GAT-1 transporter as compared with the GAT-1s of other species—even the GAT-1 of the closely related electric ray, *Torpedo*—suggest alternative interpretations. It is possible, for instance, that the skate GAT-1 protein might not be expressed in its normal configuration in *Xenopus* oocytes, leading to aberrant differences in the affinity for GABA and NO-711. In this regard it is unfortunate that no one has yet determined appropriate values for either the EC_{50} or the block by NO-711 for the skate GAT-1 in cells where the protein is normally expressed.

The Western blot performed using the GAT-1-specific antibody recognized a protein of slightly greater than 60 kDa in brain and retina. The predicted size of the skate GAT-1 protein based on its amino acid sequence is approximately 70 kDa. The reason for the difference between the predicted size as compared to that observed on the Western blot is not yet clear; proteins do not necessarily run at their predicted molecular weight, for example, if they have lipid covalently linked to the protein, or the protein may not be heavily glycosylated. For both GAT-1 and GAT-3, strong immunolabeling was detected in both the outer plexiform layer (OPL) and the inner plexiform layer (IPL). Staining extended from the inner limiting membrane to the outer limiting membrane, and prominent staining was seen surrounding many of the cell bodies in the proximal two-thirds of the inner nuclear layer (INL). This pattern of staining is consistent with the expression of GAT-1 and GAT-3 by Müller cells.

Polyclonal antibodies against GAT-1 and GAT-3 revealed significant immunoreactivity of skate Müller cells. The identity of these cells was confirmed in double-labeling experiments using an antibody against glutamine synthetase, which is known to label Müller cells of the skate and many other species (Linser et al., 1984). These histological findings are consistent with previous evidence indicating the presence of GABA transporters in Müller cells of the skate (Lam, 1975; Bruun et al., 1984; Qian et al., 1993). The immunocytochemical localization of GABA transporters in Müller cells has been investigated in a number of different species. All mammalian Müller cells thus far examined have been shown to express GABA transport proteins. In rat, Müller cells express predominantly GAT-3, with faint GAT-1 staining also detectable (Honda et al., 1995; Johnson et al., 1996). Müller cells of the guinea pig also show prominent immunolabeling for both GAT-1 and GAT-3 (Biedermann et al., 2002). Müller cells of mice express GAT-1 (Ruiz et al., 1994); studies to examine expression of other GAT subtypes have not yet been reported in this species. Hu et al. (1999) reported positive staining for GAT-3, but not GAT-1, in adult rabbit Müller cells. The Müller cells of two lower vertebrates, the tiger salamander (Yang et al., 1997) and the salmon (Ekstrom & Anzelius, 1998), displayed no positive labeling for any GAT proteins, although positive labeling was seen in other cell types. However, using a different set of antibodies, Zhao et al. (2000) reported robust immunostaining for GAT-1 and GAT-3 in the Müller cells of bullfrog. In our hands, the antibody developed against the C-terminal region of the rat GAT-1 sequence, which shows prominent immunolabeling of mammalian Müller and amacrine cells, failed to display positive staining when applied to the retina of the skate. It was this initial negative finding that prompted us to develop our own polyclonal antibody to the skate GAT-1 protein, whose cDNA had been previously cloned and characterized (Qian et al., 1998). This new antibody produced robust labeling of Müller cells while the rat GAT-1 antibody failed to label the cells. It may be worthwhile to revisit the question of whether Müller cells of other lower vertebrates express multiple GABA transport proteins using the antibody we have generated to the skate GAT-1 in the present work.

The presence of GABA transporters in amacrine cells has been reported in every species so far examined (Brecha & Weigmann, 1994; Ruiz et al., 1994; Honda et al., 1995; Johnson et al., 1996; Yang et al., 1997; Ekstrom & Anzelius, 1998; Hu et al., 1999; Zhao et al., 2000; Biedermann et al., 2002). In our own study, we note intense staining for both GAT-1 and GAT-3 in the plexiform layers, which likely represents in part Müller cell processes. The enzymatic dissociation protocol we employ to obtain isolated cells yields large numbers of photoreceptors, horizontal cells, bipolar cells, and Müller cells. However, we have not been successful in unambiguously identifying amacrine cells in these dissociations, despite applying a number of putative amacrine cell-specific markers. Our results thus do not exclude the possibility that GAT-1 and GAT-3 may also be expressed in certain classes of amacrine cells of the skate retina.

The uptake of radioactively-labeled GABA has also been observed in the horizontal cells of a number of other species, including goldfish (Ayoub & Lam, 1984), the tiger salamander (Yang & Yazulla, 1988), and the mudpuppy (Pourch et al., 1984). Moreover, electrophysiological evidence strongly supports the presence of GABA transporters in the cone-driven horizontal cells of the catfish (Cammack & Schwartz, 1993) and in horizontal cells of the all-rod skate (Malchow & Ripps, 1990; Malchow & Andersen, 2001; Kreitzer et al., 2003). Given the strong electrophysiological evidence for the transport of GABA into skate horizontal cells, it is interesting that these cells do not display positive immunolabeling for either skate GAT-1 or skate GAT-3. Our immunolabeling data thus imply that the GABA transport protein present in horizontal cells of skate is, in fact, neither GAT-1 nor GAT-3. Antibodies that recognize the skate homologues of the rat GAT-2 and BGT-1 transporters are not available at present, and it remains possible that horizontal cells express one or both of these two transporter subtypes. Alternatively, it may be that horizontal cells of the skate express a unique transporter or splice variant (i.e. one missing the epitope used to generate the anti-GAT-3 antibody) yet to be discovered.

In summary, our data demonstrate that GAT-1 and GAT-3 transporters are expressed within the skate retina and therefore likely play an important role in termination of synaptic transmission and regulation of overall GABA concentrations. The GAT-1 and GAT-3 transporters appear to be present in Müller cells of the skate, but not in horizontal cells, for which evidence of GABA transport is nonetheless strong, or bipolar cells, which do not appear to transport GABA. The observation that Müller cells of skate, and indeed the Müller cells of many mammals, express multiple GABA transporter subtypes raises a number of questions relating to specific function and regulation of each transporter that should be the focus of research in future studies.

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