Polarized Expression of GABA Transporters in Madin-Darby Canine Kidney Cells and Cultured Hippocampal Neurons*

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At least three high affinity Na⁺- and Cl⁻-dependent γ-aminobutyric acid (GABA) transporters are known to exist in the rat and mouse brain. These transporters share 50–65% amino acid sequence identity with the kidney betaine transporter which also transports GABA but with lower affinity. The betaine transporter (BGT) is expressed on the basolateral surface of polarized Madin-Darby canine kidney (MDCK) cells. Recent evidence suggests that the signals and mechanisms involved in membrane protein sorting share many functional characteristics in polarized neurons and epithelial cells. It was previously shown that the rat GABA transporter GAT-1 is located in the presynaptic membrane of axons where it plays a role in terminating GABAergic neurotransmission. When expressed in MDCK cells by transfection, GAT-1 was sorted to the apical membrane. In this report, we have localized the other two GABA transporters, GAT-2 and GAT-3, in transfected MDCK cells by GABA uptake, immunofluorescence, and cell surface biotinylation. GAT-3, like GAT-1, localized to the apical membrane of MDCK cells while GAT-2, like BGT, localized to the basolateral membrane. We have also expressed BGT in low density cultures of hippocampal neurons by microinjection and immunolocalized it to the dendrites. The distribution of GAT-3 in these neurons after transfection was axonal as well as somatodendritic. These results indicate that highly homologous subtypes of GABA transporters are sorted differently when expressed in epithelial cells or neurons and suggest that these two cell types share the capacity to distinguish among these isoforms and target them to distinct destinations.

Synaptic transmission by aminergic neurons is terminated through the uptake of the neurotransmitter by specific sodium and chloride dependent cotransporters located in the presynaptic membranes of neurons (1, 2). Following transport into the cell, the neurotransmitters accumulate in synaptic vesicles and are re-released during the next neurotransmission. GABA is an amino acid which serves as the major inhibitory neurotransmitter in the vertebrate central nervous system (3, 4). The cell surface transporter responsible for GABA re-uptake was cloned (5) and subsequently proved to be the first member of a large gene family. Other members of this family include the transporters which function in the presynaptic uptake of the biogenic amines norepinephrine (6), serotonin (7, 8), and dopamine (9–11), as well as the transporters for the amino acids taurine (12, 13), glycine (14–16), and proline (17). These transporters are proposed to span the membrane 12 times, with both amino and carboxyl termini in the cytoplasm. Another family member, the Na⁺- and Cl⁻-dependent betaine transporter, has been cloned from MDCK cells (18) and human brain (19). The canine betaine transporter is a basolateral membrane protein which protects cells in the renal medulla from hypertonicity by mediating the uptake of the osmolyte betaine (20). GABA is also a substrate for the betaine transporter. In fact, the transporter’s affinity for GABA (Kₘ ≈ 100 μM) is higher than its affinity for betaine (Kₘ ≈ 500 μM). More recently, the cDNAs for several subtypes of GABA transporters have been isolated from mouse and rat brain, bringing to four the number of GABA transporter isoforms which have been identified (21–23). Two of the high affinity (Kₘ ≈ 10 μM) rat GABA transporters, GAT-2 and GAT-3 (also known as GAT-B), share higher amino acid identity (68% and 65%, respectively) with the betaine transporter than with GAT-1 (52% amino acid identity).

Recently, we have examined the sorting behaviors of members of this family expressed by transfection in polarized epithelial cells (24). The plasma membrane of an epithelial cell is divided into an apical membrane, which frequently faces a lumen, and a basolateral membrane which is in contact with the extracellular fluid space. The protein and lipid compositions of these two membrane domains are quite different (25, 26). Neurons are also polarized cells whose plasma membranes can be divided into two domains: axons (including termini) and the somatodendritic surfaces (composed of the cell body and the dendrites). Axons are thin and usually extend great distances from the cell body while dendrites are thick at the base, tapered and shorter in length. The axonal and dendritic processes must be biochemically distinct in order for them to receive and transmit information in a highly regulated manner. Very little is known about the mechanism of generating and maintaining polarity in neurons. Dotti and Simons (27) proposed that the mechanisms involved in sorting proteins to the axonal and somatodendritic membranes are similar to those required to

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1 The abbreviations used are: GABA, γ-aminobutyric acid; GAT-1, GAT-2, GAT-3, GABA transporter subtypes; BGT, betaine transporter; MDCK, Madin-Darby canine kidney; HA, hemagglutinin; MEM, minimal essential medium; MEMF, Dulbecco’s modified Eagle’s medium; NHS-biotin, sulfo-NHS-biotin; PBS, phosphate-buffered saline.
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sort proteins to the apical and basolateral membranes, respectively, of polarized epithelial cells. When cultured hippocampal neurons were infected with the influenza fowl plague virus, vesicular stomatitis virus, these virus‘ major glycoproteins were targeted to distinct membrane domains just as they are in infected epithelial cells. The influenza hemagglutinin (HA), which is targeted to the apical membrane in MDCK cells, was predominantly sorted to the axon. In contrast, the vesicular stomatitis virus G protein, which accumulates in the MDCK basolateral membrane, was sorted to the dendrites. Furthermore, Thy-1, a glycosylphosphatidylinositol-linked protein, was sorted to the axon in hippocampal neurons (28) whereas glycosylphosphatidylinositol-linked proteins are generally present in the apical membranes of epithelial cells. The small GTP-binding phosphorylinositol-linked proteins are generally present in the more, Thy-1, a glycophosphatidylinositol-linked protein, was predominantly sorted to the axon. In contrast, the vesicular infected epithelial cells. The influenza hemagglutinin (HA), were targeted to distinct membrane domains just as they are in neurons were infected with the influenza fowl plague virus or termally, of polarized epithelial cells. When cultured hippocampal A clonal antibody (6H) against the GABA transporter protein in hippocampal neurons by microinjection and found that it accumulates in the dendrites. GAT-3, on the other hand, was found in both axons and the somatodendritic membrane. These results indicate that highly homologous subtypes of GABA transporters are distributed differently when expressed in epithelial cells and suggest that these transporters require distinct subcellular distributions in order to subserve their physiologic functions. Moreover, the apical/axonal and basolateral/dendritic sorting model proposed by Dotti and Simons (27) is consistent with the behaviors manifest by this group of membrane proteins.

EXPERIMENTAL PROCEDURES

Materials—Rabbit antisera R22 and R23, raised against amino acids 189–206 (YVEFWRNMHOMTDGLDK) and amino acids 270–288 (FYTPNRFKLSDESEVLDA), respectively, of the rat GABA transporter (GAT-1) were a generous gift from L. Edelmann and R. Jahn, Yale University, New Haven, CT. A polyclonal antiserum (anti-670) directed against the carboxyl terminus of GAT-3 (amino acids 592–608) was a generous gift from B. Kanner, The Hebrew University, Jerusalem, Israel. Anti-MAP2 polyclonal antibody was a generous gift from R. Vaille, Worcester Foundation for Experimental Biology, Worcester, MA. Anti-a 2 subunit of the Na,K-ATPase has been described (32). pCB6 and pCB6-HA were kindly provided by M. Roth, University of Texas Southwestern Medical Center, Dallas, TX. Anti-a2 subunit antibody and purine were purchased from Boehringer Mannheim. All chemicals, unless noted otherwise, were purchased from J. T. Baker, Phillipsburg, NJ.

Tissue Culture—MDCK cells were maintained in complete medium consisting of minimal essential medium (MEM), 10% fetal bovine serum (Sigma), 2 mM l-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin. Cells were grown in a 37°C humidified incubator with 5% CO2 and passaged twice weekly through exposure to 0.05% trypsin, 0.5 mM EDTA (Life Technologies, Inc.). Hippocampal neurons were isolated from 17 day rat embryos, plated on coverslips at 3000 cells/cm2, and co-cultured with glia as described previously (33). One-half of the neurons were infected with 0.9 g/liter Geneticin (Sigma), 2 mM l-glutamine, 1% -H-L-1 supplement (Ventrex Laboratories) every 4–5 days. When neurons were to be maintained for longer than 1 week, cystamine arabinoside was added to inhibit proliferation of non-neuronal cells.

Construction of c-Myc-tagged BGT-1—The c-Myc epitope EQKLISEK was added to the cystathionine cDNA of the betaine transporter cDNA (BGT-1) by the polymerase chain reaction method. BGT-1 was finally cloned into the TA cloning vector pACYC184 (New England BioLabs) which lacks BsaI and Stul restriction sites. This plasmid pACYC184-BGT1 was used as a template to synthesize a 0.8-kilobase fragment with the primers GTTGGGGAAGAGACCATGTTGGAGCAGAAGCTGATCTCTGGATGTGGCCAGC and TTGGAGCCGTGTA. The polymerase chain reaction product was cloned into the TA cloning vector pCR II (Invitrogen, San Diego, CA), digested with BsaI and Stul, and ligated to the BsaI-Stul sites of pACYC184-BGT1. The modified BGT-1 (BGT*) was finally cloned into the MuI BamHI sites of the expression vector pcB6. Plasmid DNA was amplified in competent Escherichia coli DH5α cells (gift of L. Roman). Sequencing was performed by Quest Diagnostics (Iglsen, Chatsworth, CA). The modified region of pCB6-BGT* was confirmed by dyelex sequencing.

Stable Expression of cDNAs in MDCK Cells—GAT-2 cDNA (kindly provided by L. A. Borden and K. E. Smith, Sycap Pharmaceutical, Paramus, NJ) was subcloned into the HindIII and XbaI sites of the mammalian expression vector pcB6. GAT-3 was subcloned into the Clal site of pcB6. MDCK cells were transfected in the presence of 0.1 mM chloroquine using the calcium phosphate method as described elsewhere (34–36). After about 2 weeks of selection in 0.9 μg/ml Geneticin, colonies of cells were transferred to 24-well plates using 8-mm cloning cylinders (Bellco Glass, Vineland, NJ). Subcultured cultures were scored for their ability to take up [3H]GABA (see assay below).

Transient Expression of cDNAs in COS-1 Cells—Cells were transfected using DEAE-dextran as described (37). Cells (2 × 105 per 35-mm dish) were plated on glass coverslips the night before transfection and washed twice in TBS (137 mM NaCl, 5 mM KCl, 0.7 mM CaCl2, 0.5 mM MgCl2, 0.6 mM Na2HPO4, 25 mM Tris-HCl, pH 7.4) at 37°C. To each dish, 0.5 ml of TBS containing 5 μg of DNA and 0.5 mg/ml DEAE-dextran (Pharmacia Biotech, Inc.) were added for 40 min at 37°C. The solution was discarded and the cells were incubated in 1 ml of DMEM containing 10% fetal bovine serum and 0.1 mM chloroquine for 3 h at 37°C. The cells were washed twice in TBS (37°C) and incubated in complete DMEM for 48 h before measuring protein expression.

GABA Uptake Assay—MDCK cells (4 × 105) were plated on 6.5-mm Transwell porous cell culture inserts (0.4-μm pore polycarbonate filter membrane, Costar, Cambridge, MA) 1 week before measuring GABA uptake. Assays were performed as described previously (21, 24). Monolayers were washed three times in PBS* (150 mM NaCl, 1 mM MgCl2, 0.1 mM CaCl2, 10 mM Na2HPO4, pH 7.4). [3H]GABA (2–3H)jamisobutyric acid, 30 Cl/m, DuPont NEN) in PBS* was added to either the apical (0.2 ml) or basolateral (0.5 ml) side of the filter at a final concentration of 50 nM (0.33 μCi/ml). After 10 min at room temperature, the cells were washed three times in ice-cold PBS*. Filters were excised and the cells were lysed in 200 μl of 1% SDS. A 50-μl aliquot of cell extract was removed for protein determination using the Pierce BCA protein assay reagent (Pierce) and bovine albumin as the standard. The remainder was treated with 3 ml of OptiFluor (Packard, Downers Grove, IL). Nonspecific uptake (<10%) was determined in the presence of 0.5 μM unlabeled GABA.

Cell Surface Biotinylation—Cells were grown on 24-mm Transwell inserts (0.4-μm pore, Costar) for 1 week and the medium was replaced with complete MEM (without Geneticin) containing 10 mM sodium bicarbonate. Cells were allowed to incubate for 4–24 h before the experiment. Steady-state labeling was carried out at pH 9.0 as described by Gottardi et al. (38) except that the cells were incubated with NHS-biotin for 2 × 10 min. Biotinylation at pH 9.0 was chosen because our laboratory has previously shown that many membrane proteins are more efficiently biotinylated at pH 9.0. Performing the biotinylation at pH 7.5 instead of at pH 9.0 did not alter the results. The biotinylated proteins were isolated with 100 μl of packed immobilized streptavidin and analyzed by SDS-polyacrylamide gel electrophoresis (39) and Western blotting (40) as described previously (34, 38). The blot was incubated with R22 (1:200) or 6H (1:500...
primary antibodies followed by peroxidase-conjugated secondary antibodies (Sigma) and developed with Enhanced Chemiluminescence reagent (Amersham).

Microinjection—DNA solutions (0.5–1 μg/μl, pH 7.0) were passed through a 0.22-μm Spin-X membrane (Costar) and verified on an ethidium bromide containing agarose gel. Lysine-fixable fluorescein- or Texas Red-labeled molecular probes (Molecular Probes) was added to the DNA at a final concentration of 10 μg/ml, 2.5 mM Tris-HCl, pH 7.5. Injection pipettes were pulled from Narishige model GD-1 microcapillary tubes to an approximate diameter of 0.5 μm. The pipette was filled through the larger opening with 2.5 μl of solution using a microinjector (Bio-Rad). Each coverslip with neurons attached was transfected, cell side up, to a 35-mm dish containing 1.5 ml of fresh neuronal medium supplemented with 20 μM NaHepes, pH 7.4. The dish was placed on the stage of an inverted microscope (Olympus) and a filled pipette was positioned vertically over the cells. The cells were injected manually using a micromanipulator (Narishige). Injection was indicated by swelling of the nucleus and change in light reflection. Fifty to one hundred cells were injected per coverslip over a period of 15 min at room temperature. Dishes were kept in a humidified 37 °C incubator until the injections were completed (up to 3 h). The coverslips were returned, cell side down, to glial cultures in conditioned medium for 48 h.

Immunofluorescence—MDCK cells were grown on 9-mm Falcon culture inserts (0.45 μm pore size, Cyclophil membrane, Becton Dickenson Labware, Lincoln Park, NJ) or 24-mm Transwell inserts (0.4 μm pore size) and processed for indirect immunofluorescence (34). All primary and secondary antibodies and Texas Red avidin were diluted in GSDB (16% goat serum (Sigma), 0.3% Triton X-100, 0.45 mM NaCl, 20 mM NaP, pH 7.4; Ref. 30). Incubations were carried out at room temperature in a humidified, light-protected box. MDCK cells were rinsed in PBS and fixed in methanol (−20 °C) for 10 min or in 3% paraformaldehyde for 25 min. After washing in PBS, the cells were incubated for 15 min in 0.3% Triton X-100, 0.1% bovine serum albumin (Sigma) in PBS (Buffer A), and blocked for 30 min in GSDB. Next, the cells were incubated with anti-GABA transporter (R22 or anti-670, diluted 1:50) and anti-Na,K-ATPase α subunit (6H, 1:100) antibodies for 1 h, followed by three washes (5 min each) in Buffer A. The cells were then incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Sigma) and rhodamine-conjugated anti-mouse IgG (Boehringer Mannheim), both diluted 1:100, for 1 h and washed again in Buffer A. Hippocampal neurons were rinsed twice briefly in PBS (37 °C), fixed in freshly prepared 4% paraformaldehyde in PBS, washed three times (5 min each) in 10 mM glycine in PBS, and once more in PBS. Cells were permeabilized in Buffer A for 15 min, blocked in GSDB for 30 min, blotted in avidin solution (Vector Laboratories, diluted 1:1 with 2X GSDB) for 15 min, rinsed in PBS for 5 min, and blocked in biotin solution (diluted 1:1 with 2X GSDB) for 15 min. The cells were incubated with anti-c-Myc (Oncogene Scien, 1:50) or anti-α-HA (1:200) monoclonal antibodies for 1 h. Double labeling was carried out by incubation with anti-MAP2 (1:100) or synapsin (1:50) polyclonal antibodies in the same primary antibody solution. The cells were washed three times (5 min each) in Buffer A, then incubated with 10 μg/ml biotinylated anti-mouse IgG (Vector) and Cy5-conjugated anti-rabbit IgG (Jackson Immunoreagents, 1:100) for 1 h. The washed cells (3 × 5 min in Buffer A) were incubated in Texas Red-conjugated avidin (Molecular Probes, 1:200) for 30 min and washed again. All specimens were washed for 10 min in 5 mM NaP, pH 7.5, before mounting. Coverslips were mounted cell side down in a freshly prepared solution of 0.1% p-phenylenediamine (Sigma), 70% glycerol, 150 mM NaCl, 10 mM NaP, pH 7.4, or in Vectashield mounting solution (Vector Laboratories, Burlingame, CA) and sealed with nail polish. Filters were mounted cell side up and coverslipped. The cells were initially examined using a Zeiss Axiovert 135 microscope. Immunofluorescence and phase-contrast images were photographed using TMX100 or 400 film (Eastman Kodak Co., Rochester, NY). Confocal images were generated on a Zeiss laser scanning microscope using the following excitation/emission wavelengths: 488 nm/515–560 nm (fluorescein), 568 nm/590–640 nm (Texas Red), 667 nm/650–710 nm (Cy5). Confocal z-axis sections were generated using a 0.2-μm motor step. All images are the product of 8-fold line averaging. The green channel showed no significant bleed-through from the fluorescein to rhodamine/Texas Red channels since many of the cells which were strongly labeled with fluorescein had no rhodamine or Texas Red labeling.

RESULTS

GAT-2 and GAT-3 Are Expressed on Opposite Membrane Domains in MDCK Cells—The GABA transport activity was catalyzed by the different MDCK cell lines expressing GAT-2 or GAT-3 are shown in Fig. 1. Five positive clones were isolated from each transfection. Cells transfected with GAT-2 transported GABA 3–10 times faster when the labeled substrate was added to their basolateral surfaces as opposed to their apical surfaces. Cells transfected with GAT-3 transported GABA rapidly only when GABA was added to their apical surfaces. Therefore, these two GABA transporters are functionally expressed in opposite membranes in MDCK cells. Cell lines GAT2470 and GAT348 were used in subsequent experiments.

We wished to confirm the apparent polarized distributions of these transporters using a method to localize the expressed proteins which is independent of their transport capacities since functional assays only report the distribution of the active populations. We chose, therefore, to complement the transport assay with antibody based detection methods. Two polyclonal antibodies (R22 and R23) raised against peptides from GAT-1 were tested for their ability to cross-react with GAT-2 and GAT-3. In order to express high levels of transporter for antibody characterization studies, we transiently transfected COS-1 cells with GAT-2 or GAT-3 cDNAs and measured antibody binding by indirect immunofluorescence. R22 recognized GAT-2 and GAT-3 in COS-1 cells (Fig. 2, a and b); however, the intensity of fluorescence was considerably lower for the GAT-3 transfected COS-1 cells (Fig. 2b). Antibody R23 recognized neither GAT-2 nor GAT-3 (not shown). Although cell surface labeling was clearly visible, most of the transporters were localized intracellularly, probably in the endoplasmic reticulum and Golgi apparatus. This pattern of staining is often seen in COS-1 cells induced to overexpress membrane proteins. The GAT-3 specific antibody, anti-670, labeled the surface as well as intracellular membranes of COS-1 cells expressing GAT-3 (Fig. 2c). This antibody did not react with cells expressing GAT-2 (not shown).

When stably transfected MDCK cells expressing GAT-2 were labeled with R22, a lateral staining pattern was observed (Fig. 3, A and C), similar to the pattern obtained with an antibody against the Na,K-ATPase, a known basolateral protein (Fig. 3, B and D). We discovered that MDCK cells transfected with GAT-3 did not stain with R22, so anti-670 had to be Cut off to fit the page.
used for immunofluorescence. With the latter antibody, we observed bright staining of the apical membrane of MDCK cells transfected with GAT-3 (Fig. 3, E and G) while the Na,K-ATPase was distributed basolaterally in the same cells (Fig. 3, F and H).

Finally, we performed cell surface biotinylation experiments to confirm the polarity of GAT-2 and GAT-3 in MDCK cells. Fig. 4A shows that GAT-2, an 85-kDa protein, was accessible to biotinylolation predominantly from the basolateral side of intact MDCK cells. In contrast, GAT-3 was preferentially biotinylated from the apical side. The Na,K-ATPase was biotinylated from the basolateral surface in all three cell lines (Fig. 4B), indicating that these cells are properly polarized. These data are consistent with the transport and microscopy results. We conclude, therefore, that GAT-2 behaves as a basolateral protein when expressed by transfection in MDCK cells, whereas its homologue GAT-3 accumulates in the apical plasma membrane.

Epitope-tagged Betaine Transporter Is Basolateral in MDCK Cells—The data presented by Pietrini et al. (24) and the work presented here describe the sorting behavior of all four GABA transporters in MDCK cells. So far, however, only GAT-1 has been localized to a specific subcellular domain in neurons. We wondered whether neurons would recognize the basolateral sorting information associated with the betaine transporter (BGT) and target this protein to the dendritic membrane. In order to detect the expression of BGT by immunofluorescence after transfection, we added a c-Myc epitope to its carboxyl terminus and used an anti-c-Myc antibody to stain the cells. Fig. 5 shows xy and xz confocal sections of MDCK cells stably transfected with the c-Myc-tagged betaine transporter (BGT*). BGT*, like endogenous betaine transporter (20) and untagged BGT expressed by transfection (24), was expressed on the basolateral surface of polarized MDCK cells. Therefore, the addition of the 10-amino acid epitope did not interfere with the proper folding and sorting of this transporter.

Expression of the Betaine Transporter in Hippocampal Neurons—We wished to determine if members of the GABA transporter family would occupy different membrane domains when synthesized in polarized neurons. The betaine transporter is not endogenously expressed in rat neurons (41). Thus, we transfected cultured hippocampal neurons by microinjecting the cDNA directly into their nuclei and visualized the expressed protein by immunofluorescence microscopy. Table I summarizes the data on the success of the injection protocol. There was considerable day to day variation in the transfection efficiency. In one experiment, 10 out of 300 injected cells were positively labeled, while in another experiment only one cell (out of 300 injected) expressed the transporter. Fig. 6 shows a neuron that has been double-labeled with antibodies to c-Myc (BGT*) and the dendritic marker, MAP2 (42). Nearly all the processes labeled with anti-c-Myc (BGT*) were also positive for MAP2. It would appear, therefore, that the betaine transporter was selectively expressed in the dendrites of hippocampal neurons. Several MAP2 negative processes (arrows in the phase-contrast micrograph) were devoid of betaine transporter, but these axons may belong to neighboring uninjected cells. To avoid this ambiguity, we co-injected BGT* cDNA with lysine-fixable fluorescein/dextran. The fluorescein/dextran was small enough to pass through the nuclear membrane and label all the processes of the injected cell (Fig. 7, DEX) without affecting the biosynthesis or targeting of the proteins. In the neuron shown in Fig. 7, it is clear that the fluorescein-labeled axon (arrow in DEX) originates from the injected cell and does not express the betaine transporter (BGT*).

Some of the injected cells were double-labeled with anti-c-Myc and an antibody against the synaptic vesicle protein, synapsin (43). Synapsin was found in the axons (of both injected
and un.injected cells) and appeared as bright spots at sites of synaptic contact with the cell body and dendrites (Fig. 7, SYN). The arrowheads point to processes which express BGT*. Three of the six short BGT*-labeled processes are most likely dendrites since they do not contain synapsin. The other three processes are labeled by synapsin but they could be dendrites in contact with neighboring axons. Expression of HA in Hippocampal Neurons by Microinjection—To demonstrate that the somatodendritic distribution of the betaine transporter is not an artifact of the microinjection method, we also microinjected neurons with the cDNA for influenza hemagglutinin (HA), a membrane protein which has previously been shown to localize to the axons of cultured hippocampal neurons infected with the influenza virus (27). Cells injected with HA cDNA had a staining pattern very different from those injected with BGT* (Fig. 8). Although the cell body and some dendrites were labeled with HA antibody, the axon was also labeled. The labeled processes often extended more than 200 μm from the cell body and formed multiple loops, as is characteristic for axons of cultured neurons. Such axonal labeling was not observed in neurons microinjected with BGT*. GAT-3 Is Expressed in the Axons of Microinjected Hippocampal Neurons—When cultured hippocampal neurons were

| Injection solution | Total number of cells injected | No. of cells containing FL/dextran | No. of cells expressing protein | n |
|--------------------|-------------------------------|----------------------------------|--------------------------------|---|
| pCB6               | 600                           | N/A                              | 0                              | 2 |
| pCB6-BGT*          | 1710                          | N/A                              | 39                             | 6 |
| pCB6-HA            | 1090                          | N/A                              | 14                             | 3 |
| pCB6-BGT* + fluorescein/dextran | 450                      | 44                              | 11                             | 2 |
| pCB6-GAT3 + fluorescein/dextran | 665                      | 39                              | 17                             | 3 |

*Experimental Procedures.* The total number of cells injected, the number of cells containing fluorescein (FL)/dextran, and the number of cells expressing protein by immunofluorescence are listed. N/A = not applicable; n = number of experiments, each performed on a different day.
stained with an antibody specific for GAT-1, a subset (~10%) of the cells were labeled (24). To determine if GAT-3 is also endogenously expressed, hippocampal neurons were stained with the GAT-3 specific antibody, anti-670. We were unable to see any positive staining above the background. Therefore, GAT-3 cDNA was microinjected into these neurons to determine if the apical membrane sorting information in GAT-3 specifies an axonal localization. Fig. 9 shows a typical neuron expressing GAT-3 in both the axon and dendrites. This expression pattern resembles that of HA and is quite distinct from that of the somatodendritically localized BGT*.

These results suggest that certain proteins (e.g. betaine transporter) are restricted to the somatodendritic membrane while others (HA and GAT-3) are expressed in both somatodendritic and axonal membranes. Moreover, apical membrane proteins appear to be transported to axons while basolateral membrane proteins remain in the somatodendritic membrane when expressed in neurons.

**DISCUSSION**

We have previously shown that two members of the neurotransmitter transporter gene family, GAT-1 and the betaine transporter, are sorted to two different membrane domains in MDCK cells (24). It should be noted that the betaine transporter can also transport GABA but with a lower affinity. In this report, we show that the two most recently cloned GABA transporters, GAT-2 and GAT-3, are also differentially localized in MDCK cells according to both functional and biochemical criteria. GAT-3, like GAT-1, was expressed on the apical surface of transfected MDCK cells. GAT-2, like the betaine transporter, was expressed mainly on the basolateral surface of MDCK cells. The construction of chimeric proteins consisting of sequences from GAT-3 and GAT-2, which share ~65% sequence identity, should help us to determine the sorting signals which mediate the subcellular targeting of members of this family of proteins.

In the context of our data on sorting, it is interesting to consider the tissue specific expression of the GABA transporter isoform mRNAs by Northern blot analysis. GAT-1 and GAT-3 are expressed in brain and retina, while GAT-2 mRNA is found in liver and kidney as well as the brain and retina (21). It would appear, therefore, that both of the exclusively neuronal forms of the GABA transporter behave as apical proteins, while the isoforms endogenously expressed in epithelia are primarily basolateral. The functional significance of these non-neuronal GABA transporters is not known. Presumably, however, epithelial GAT-2 may function like the betaine transporter to import a solute from the serum. Thus, we expect that this protein is basolaterally disposed in the tissues which express it endogenously.

To study the sorting mechanism in neurons, we have developed a method of transfecting primary cultures of embryonic hippocampal neurons. Primary cultures of neurons are difficult to transfect with DEAE-dextran, calcium phosphate, or cationic lipids, methods commonly used to transfet other mammalian cells in culture. High efficiency transfection of neurons has been accomplished using Herpes Simplex or Semliki Forest viral vectors (44, 45); however, there are problems associated with introducing viral components. Hippocampal neurons do not survive for more than 8 h following viral infection (45) and may be subjected to alterations of cellular physiology which could affect the polarized sorting of neuronal proteins. For example, in cultured dorsal root ganglion and spinal cord neurons, the normally somatodendritic MAP2 appears in axons after rotavirus infection (46). The introduction of foreign DNA into the nucleus of neurons by microinjection should cause little damage to the cells since the plasma membrane is expected to immediately re-seal by a calcium-dependent mechanism following puncture (47).

We expressed the renal betaine transporter by microinjection of cultured hippocampal neurons and found that this basolateral membrane protein is sorted exclusively to the somatodendritic membrane. Next, we expressed influenza hemagglutinin by microinjection and found that, unlike the betaine transporter, this protein was also delivered to axons. Finally, we expressed the GABA transporter GAT-3 in hippocampal neurons and noted that, like HA, it was transported to the axon as well as being present in the cell body and dendrites. GAT-3 would be expected to reside in presynaptic membranes if its role in the brain, like that of GAT-1, involves neurotransmitter uptake from the synaptic cleft following neu-
rotransmission. The subcellular distribution of GAT-2 in neurons has yet to be determined. However, in light of its preference for the basolateral membrane when expressed in MDCK cells, we can predict that GAT-2 will be sorted like BGT, i.e. to the dendrites.

Our results are consistent with the hypothesis that apical and basolateral sorting signals are interpreted as axonal and dendritic targeting signals, respectively, by hippocampal neurons. While the betaine transporter was expressed exclusively in the somatodendritic membrane, influenza HA and GAT-3 were less polarized. The latter two proteins were found in dendrites as well as axons. Dotti and Simons (27) also found some HA in the dendrites of influenza-infected neurons.

FIG. 7. Expression of the betaine transporter in neurons co-injected with pCB6-BGT* and fluorescein/dextran. Neurons were injected after 8 days in culture and double-labeled for the betaine transporter (BGT*) and synapsin (SYN). Fluorescein/dextran (DEX) labeled the axon (indicated by arrows) and most dendrites (arrowheads) of the injected cell. Synapsin is found in the synaptic vesicles of axons (43). Sites of synaptic contact between the axon and somatodendritic membrane are indicated by bright spots which are characteristic of synapsin labeling (SYN). These as well as other dendrites which are devoid of synaptic contacts expressed the betaine transporter (BGT*). The axon (arrows) did not express the transporter.

FIG. 8. Expression of hemagglutinin in the axons of cultured hippocampal neurons by microinjection. Neurons after 17 days in culture were microinjected with pCB6-HA and double-labeled with anti-HA (HA) and anti-MAP2 (MAP2) antibodies. The arrow indicates the axon which is negative for MAP2. HA was found in the axon in addition to being present in the cell body and dendrites. The insets show another cell whose thin axon (arrows) runs along the edges of the main dendrite (labeled with MAP2; arrowheads). Bar, 15 μm.

FIG. 9. Expression of GAT-3 in the axons of cultured hippocampal neurons. Neurons after 8 days in culture were co-injected with pCB6-GAT3 and fluorescein/dextran. The cells were double-labeled with anti-670 (top left panel) and monoclonal anti-MAP2 (bottom left panel) antibodies followed by rhodamine-conjugated anti-rabbit IgG (GAT3) and Cy5 anti-mouse IgG (MAP2). Immunofluorescence was examined by confocal microscopy. GAT-3 is expressed in the long, thin axon (arrows) originating from the injected cell marked by fluorescein/dextran (DEX). MAP-2 positive dendrites are indicated by arrowheads. Bottom right, phase-contrast micrograph of same cell shown in the confocal images.
ensuring the correct function of these transporters. Cellular distribution almost certainly plays an important role in epithelial cells and neurons. Attainment of the proper subcellular distribution will be required to understand fully the similarities and differences relating sorting in these cell types.

Our data, taken together with that of Pietrini et al. (24), demonstrate that the four members of a highly homologous family of transport proteins are differentially distributed in epithelial and neuronal cells. Attainment of the proper subcellular distribution almost certainly plays an important role in ensuring the correct function of these transporters in situ. It will be important, therefore, to identify the sorting information embedded in these proteins and to determine its role in their function. Once these sorting signals are determined, the techniques presented here can be employed to ascertain whether the same signals are recognized by the neuronal and epithelial sorting machinery.

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