Identification of Differentiation-Associated Brain-Specific Phosphate Transporter as a Second Vesicular Glutamate Transporter (VGLUT2)

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Glutamate is the major excitatory neurotransmitter in mammalian CNS. In the presynaptic nerve terminal, glutamate is stored in synaptic vesicles and released by exocytosis. Previously, it has been shown that a transport protein originally identified as a brain-specific Na^+-dependent inorganic phosphate transporter I (BNPI) functions as vesicular glutamate transporter and thus has been renamed VGLUT1. Recently, a protein highly homologous to VGLUT1, "differentiation-associated BNPI" (DNPI), has been discovered. Northern blot and *in situ* hybridization analyses indicate that DNPI mRNA is expressed in some brain regions in which VGLUT1 mRNA is not expressed. We now show that DNPI functions as vesicular glutamate transporter with properties very similar to VGLUT1 and propose to rename the protein VGLUT2. VGLUT2 is highly enriched in synaptic vesicles. Furthermore, VGLUT2 resides on a vesicle population that is distinct from vesicles containing the vesicular GABA transporter or VGLUT1, showing that the expression of VGLUT1 and VGLUT2 do not overlap. When VGLUT2 was expressed in BON cells, membrane fractions displayed ATP-dependent, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-sensitive glutamate uptake. Overexpression of VGLUT2 in cultured autaptic GABAergic neurons yielded postsynaptic currents that were insensitive to the $GABA_A$ receptor antagonist bicuculline but blocked by the AMPA-receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[F]quinoxaline. Thus, expression of VGLUT2 suffices to cause GABAergic neurons to release glutamate in addition to GABA in a manner very similar to that reported previously for VGLUT1.

Key words: glutamate; GABA; synaptic vesicle; uptake; vesicular transporter; autapse

Classical (nonpeptide) neurotransmitters are loaded into synaptic vesicles before they are released from presynaptic terminals. Uptake is mediated by specific vesicular neurotransmitter transporters that depend on an electrochemical proton gradient as driving force and that are different from their Na^+ gradient-dependent counterparts at the plasma membrane (Masson et al., 1999). In recent years, most of vesicular neurotransmitter transporters have been identified and characterized at the molecular level (Reimer et al., 1998). These include the vesicular monoamine transporters (VMAT1 and VMAT2), the vesicular acetylcholine transporter (VAChT), and the vesicular GABA transporter [VGAT (also termed VIAAT)]. Recently, a transport protein originally characterized as brain-specific Na^+-dependent inorganic phosphate (Pi) transporter I (BNPI) (Ni et al., 1994) has been shown to operate as vesicular glutamate transporter and is now referred to as VGLUT1 (Bellocchio et al., 2000; Takamori et al., 2000b). Although injection of VGLUT1/BNPI mRNA into *Xenopus* oocytes significantly increased Na^+-dependent Pi uptake (Ni et al., 1994), several lines of evidence suggests that its true function is to load synaptic vesicles with glutamate. First, VGLUT1 expression is confined to subpopulations of axon terminals supposed to be glutamatergic where it is exclusively localized on synaptic vesicles (Bellocchio et al., 1998; Takamori et al., 2000b). Second, VGLUT1-containing synaptic vesicles immunoisolated from rat brain are enriched in glutamate uptake activity but display only scant GABA uptake activity, whereas VGAT-containing vesicles show the opposite pattern. Third, membrane fractions isolated from a VGLUT1-expressing neuroendocrine cell line exhibit carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)-sensitive glutamate uptake activity with properties similar to glutamate uptake by purified synaptic vesicles (Bellocchio et al., 1998; Takamori et al., 2000b). Fourth, VGLUT1-expressing cells released glutamate in a quantal manner, which was monitored by reporter cells expressing a nondesensitizing variant of the AMPA receptor. Fifth, GABAergic neurons kept in autaptic culture release glutamate in addition to GABA when VGLUT1 is exogenously expressed. Thus, expression of VGLUT1 suffices to convert nonglutamate releasing cells to glutamate releasing cells (Takamori et al., 2000b).

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Despite the functional evidence that VGLUT1 functions as a glutamate transporter in glutamatergic neurons, VGLUT1 is missing from many excitatory synaptic pathways that are thought to use glutamate as transmitter (Bellocchio et al., 1998). Thus, it is possible that there are other vesicular glutamate transporters in addition to VGLUT1. Recently, a protein homologous to VGLUT1, "differentiation-associated BNPI" (DNPI), was cloned by a differential display technique as a gene that is upregulated when rat pancreatic AR42J cells are differentiated into a neuron-like phenotype by a combination of activin A and betacellulin (Aihara et al., 2000). Sequence comparison revealed a high degree of similarity between VGLUT1 and DNPI (82% identity at the amino acid level). Furthermore, DNPI expression, like VGLUT1 expression, in *Xenopus* oocytes enhanced Na^+-dependent phosphate uptake activity. Interestingly, Northern blot and *in situ* hybridization demonstrated that DNPI mRNA is predominantly expressed in brain regions (medulla, substantia nigra, subthalamic nucleus, and thalamus) in which the expression of VGLUT1 mRNA is low or undetectable (Aihara et al., 2000; Hisano et al., 2000), suggesting that DNPI may instead function as the vesicular glutamate transporter. Here we show that this is indeed the case and that DNPI (now renamed VGLUT2) exhibits functional properties very similar to VGLUT1.

MATERIALS AND METHODS

Antibodies and plasmids. Antisera were raised in rabbits against a fusion protein containing glutathione *S*-transferase (GST) and the amino acids (aa) 510–582 of rat DNPI, aa 464–582 of DNPI, and aa 456–561 of rat VGLUT1 produced and purified from *Escherichia coli* using standard procedures. Rabbit antibodies raised against synthetic peptides of VGLUT1 (VGLUT1/N2) and VGAT (VGAT/1) and antibodies against synaptophysin and the 116,000 subunit of the vacuolar proton pump were described previously (Takamori et al., 2000a,b).

For transfection experiments, an *Eco*RI fragment derived from human DNPI cDNA in pGEM3zf(+) (kind gift from Jun Takeda, Gunma University, Gunma, Japan) (Aihara et al., 2000) was subcloned into pIRES2-EGFP (Clontech, Palo Alto, CA) at the *Eco*RI site, and the direction of the insert was confirmed by restriction enzyme digestion. For Semliki Forest virus construct, the *Eco*RI fragment of DNPI was first subcloned into pcDNA3.1 (Invitrogen, San Diego, CA). A *Bam*HI/*Pme*I fragment derived from it was subcloned again into pcDNA3.1 together with *Eco*RV/*Not*I fragment of IRES-EGFP (a kind gift from Jens Rettig, University of Homburg, Homburg, Germany). The *Pme*I fragment containing DNPI-IRES-EGFP was finally cloned into pSCA1 (DiCiommo and Bremner, 1998) at *Sma*I site. Virus was produced by transfecting 10 μg of DNPI-IRES-EGFP-pSCA1 and pSCA-Helper 1 in HEK cells by the calcium phosphate method.

Subcellular fractionation and immuno-isolation. Affinity-purified antibodies against VGLUT1 and VGAT (VGAT/1) were conjugated to Eupergit C1Z methacrylate microbeads as described previously (Burger et al., 1989; Takamori et al., 2000a,b). Synaptophysin beads and control beads (glycine-inactivated) were prepared as described previously (Burger et al., 1989). The LP2 fraction from rat brain (starting material) was incubated with beads for 2 hr at 4°C with constant rotation. After incubation, the beads were sedimented by centrifugation at 10,000 rpm for 1 min and washed three times with PBS. Unbound membranes in the supernatant solution and the same amount of starting material were pelleted at 80,000 rpm for 20 min in a TLA120.2 rotor. All membranes or bead pellets were resuspended in SDS-PAGE sample buffer for gel electrophoresis. Synaptic vesicles were purified according to a conventional procedure described previously (Hell and Jahn, 1994).

Generation of BON stable transfectants. BON cells, a human pancreatic tumor cell line, were cultured in DMEM/nutrient mix F-12 (1:1), supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Twenty micrograms of human DNPI-pIRES2-EGFP were used for transfecting cells by means of the calcium phosphate method (Chen and Okayama, 1987). The transfected cells were then selected in the presence of 800 μg/ml G418, and the resulting clones were screened for EGFP fluorescence by fluorescence microscopy. DNPI expression in EGFP-positive clones was confirmed by immunofluorescence and by immunoblotting using the DNPI-specific antiserum. Two clonal lines were established (DNPI-8 and DNPI-10) and used for glutamate uptake assay.

Glutamate uptake assay. Glutamate uptake assay using isolated membrane from BON cells was performed as described previously (Takamori et al., 2000b). Briefly, cultured BON cells were washed twice with ice-cold PBS and then harvested in 0.32 M sucrose and 4 mM HEPES-NaOH, pH 7.4. The cells were homogenized, and nuclei, large membranes, and cell debris were cleared by centrifugation at 10,000 × *g* for 5 min. The supernatant was sedimented by centrifugation at 200,000 × *g* for 20 min in a TLA120.2 rotor (Beckman Instruments, Fullerton, CA). The resulting pellet was resuspended in standard uptake assay buffer (0.32 M sucrose, 4 mM KCl, 4 mM $MgCl_2$, and 10 mM HEPES-KOH, pH 7.4). Fifty micrograms of membrane were preincubated at 32°C, and the reactions were started by adding 40 μM glutamate containing 2 μCi [^{3}H]glutamate (NEN, Boston, MA) in the presence of 2 mM ATP. The reactions were stopped by adding 3 ml of ice-cold assay buffer and were then rapidly filtered, followed by an immediate wash with 3 ml of ice-cold assay buffer twice. Proton uncoupler FCCP (46 μM final) was added to the reaction mixture to measure background activity where indicated.

Hippocampal autaptic culture and neurophysiology. Hippocampal mouse neurons were cultured on microislands using standard procedures (Bekkers and Stevens, 1991). Astrocytes feeder layers were grown to confluency in minimal essential medium supplemented with 10% fetal calf serum. Neurons were plated in serum-free medium (Neurobasal medium A+B27; Life Technologies, Gaithersburg, MD). Experiments from inhibitory neurons were performed at 10–20 days *in vitro* and 36–48 hr after addition of the Semliki Forest virus. Inhibitory cells were selected according to their cellular morphology and were positively identified by the postsynaptic current shape and its block by bicuculline. The standard extracellular medium contained (in mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 $CaCl_2$, and 4 $MgCl_2$. The osmolarity was 300 mOsm, and the pH was 7.3. Solutions were applied using an array of quartz flow pipes positioned within 100–200 μm of the neuron and connected to gravity-fed reservoirs. Each flow pipe was controlled by solenoid valves and was moved with a piezoelectric device under the control of computer software (Rosenmund et al., 1995). Patch pipette (borosilicate) resistance was 2–3.5 MΩ. Pipette solutions for neurons included: 120 mM KCl, 10 mM HEPES, 1 mM EGTA, 4.6 mM $MgCl_2$, 4 mM Na_4ATP, 15 mM creatinephosphate, and 50 U/ml creatinephosphokinase. The pH was 7.3, and the osmolarity was 300 mOsm. Currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Series resistance was 60–90% compensated, as was cell capacitance (5–25 pF). Electrophysiological data were acquired on a IBM 586 clone (pClamp 8; Axon Instruments) and analyzed on a Macintosh computer (AXOGRAPH4; Axon Instruments). The acquisition rate was 10 kHz, and data were filtered at 2 kHz. Data are expressed as mean ± SE.

Others. SDS-PAGE was performed according to Laemmli's method (Laemmli, 1970), and immunoblotting was performed according to Towbin et al. (1979). For detection, the appropriate secondary antibody or Protein A (both conjugated to horseradish peroxidase; Sigma, St. Louis, MO) was used. After washing steps, the horseradish peroxidase was detected by enhanced chemiluminescence using a commercially available kit (Pierce, Rockford, IL).

RESULTS

VGLUT2/DNPI is localized to synaptic vesicle populations distinct from VGLUT1- and VGAT-containing vesicles

To compare the subcellular distributions of VGLUT2/DNPI and VGLUT1, we raised antisera using the bacterially expressed C-terminal tail regions, fused to GST, as antigen. Three new rabbit sera were obtained, two using different constructs for VGLUT2/DNPI and one for VGLUT1. As shown in Figure 1*A*, the two antisera raised against VGLUT2/DNPI recognized a single band of 65 kDa in enriched synaptic vesicles (LP2) obtained from adult rat brain. In contrast, the serum raised against the tail domain of VGLUT1 recognized a band with higher mobility (apparent M_r of 60,000). This band is indistinguishable from the band recognized by a VGLUT1 antiserum raised against an N-terminally located peptide as described previously (Taka-

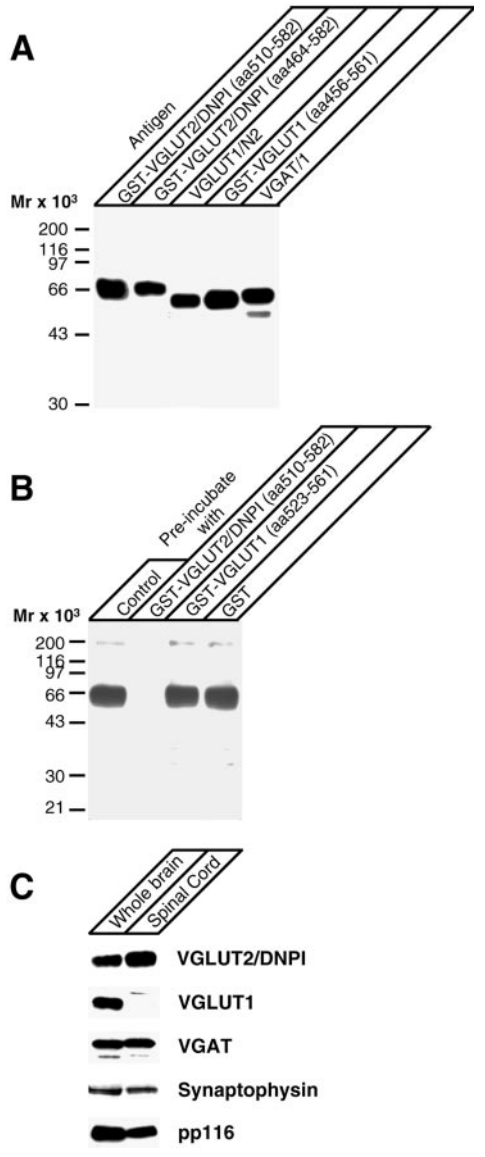

Figure 1. Characterization of antibodies specific for VGLUT2/DNPI and VGLUT1 by immunoblotting using a rat brain membrane fraction enriched in synaptic vesicles (LP2). *A*, Rabbit antisera raised against rat VGLUT2/DNPI and VGLUT1 fusion proteins recognize distinct bands. The portion of each protein fused to GST is given in *parentheses*, based on the rat amino acid sequences. All VGLUT2/DNPI antibodies recognize a single band of 65 kDa, whereas the serum raised against rat VGLUT1 recognized a band with higher mobility (60 kDa), giving an identical signal to a peptide-specific serum described previously (Takamori et al., 2000b). For comparison, a blot for VGAT was performed in parallel. *B*, VGLUT2/DNPI and VGLUT1 antisera are specific for their respective antigens (antibody raised against GST-VGLUT2/DNPI aa 510–582). The immunoreactive band recognized by the VGLUT2/DNPI serum was completely abolished when 10 μl of antiserum was preincubated with the antigen used for immunization. In contrast, no inhibition was observed when the serum was preincubated with GST-VGLUT1 (aa 523–561) or with GST. *C*, VGLUT2/DNPI but not VGLUT1 is detected on synaptic vesicles (LP2 fraction) of rat spinal cord, demonstrating that the VGLUT1 antiserum does not cross-react with VGLUT2/DNPI. *pp116*, The 116,000 subunit of the vacuolar proton pump.

mori et al., 2000b). All signals were abolished when the sera were preincubated with their respective antigens. In contrast, no signal reduction was observed when the VGLUT2/DNPI-specific sera were preincubated with the VGLUT1 fusion proteins or with GST alone and vice versa (Fig. 1*B*, and data not shown). To further confirm that our VGLUT1 serum does not cross-react with VGLUT2/DNPI, we analyzed enriched vesicle fractions isolated from brain with those isolated from spinal cord. Only VGLUT2/DNPI was detectable in the spinal cord fraction (Fig. 1*C*), in contrast to the brain fractions that contain both isoforms, in good agreement with a previous study (Hisano et al., 2000). Together, these data confirm that all new sera are specific for their respective antigens, with no cross-reactivity between VGLUT1 and VGLUT2/DNPI. Unless indicated otherwise, the serum obtained after immunization with the shorter fragment (aa 510–582) was used for the detection of VGLUT2/DNPI.

First, we examined whether VGLUT2/DNPI cofractionates with synaptic vesicles during purification of synaptic vesicles from rat brain. When subcellular fractions were analyzed by immunoblotting, VGLUT2/DNPI copurified with VGLUT1, VGAT, and the vesicle marker synaptophysin, with the highest enrichment in the purified vesicle fraction (Fig. 2*A*). In contrast, plasma membrane proteins (plasma membrane glutamate transporter EAAC1, NMDA receptor; data not shown) (Renick et al., 1999), were lost during the purification steps, indicating that DNPI is predominantly expressed on synaptic vesicles rather than plasma membrane.

Second, we investigated whether VGLUT2/DNPI is present on vesicles specific for other classical neurotransmitters, particularly GABA. We took advantage of a recently established immunoisolation procedure that allows for the isolation of GABA-specific synaptic vesicles with the aid of immobilized antibodies specific for the cytoplasmic tail domain of VGAT. The isolated vesicles are enriched in GABA uptake activity and are devoid of other vesicular neurotransmitter transporters, such as VGLUT1, VMAT2, and VAChT (Takamori et al., 2000a). As shown in Figure 2*B*, VGLUT2/DNPI, like VGLUT1, did not coenrich with VGAT on the VGAT beads, whereas almost all VGAT-containing vesicles were bound. Rather, quantitation revealed that $<10\%$ of the starting amounts (input) of both VGLUT1 and VGLUT2/DNPI were bound to VGAT beads under these conditions, indicating that VGLUT2/DNPI is present on a vesicle population distinct from GABA-transporting vesicles.

Next, we asked whether VGLUT1 and VGLUT2/DNPI are coexpressed on the same, or at least an overlapping, population of synaptic vesicles. Previous work has shown that the two molecules differ in their distribution within the CNS, but it is not known whether both transporters may be present on the same vesicles. Therefore, we immuno-isolated vesicles using an antibody specific for VGLUT1 and tested them for the presence of VGLUT2/DNPI. As shown in Figure 2*C*, VGLUT1-containing vesicles lack VGLUT2/DNPI. In contrast, vesicles immuno-isolated from the same starting material using antibodies for the general vesicle marker synaptophysin contain both transporters. These results demonstrate that VGLUT1 and VGLUT2/DNPI reside on different vesicle populations and are consistent with previous observations showing differential distribution of these transporters by *in situ* hybridization and by immunohistochemistry (Hisano et al., 2000; Sakata-Haga et al., 2001).

VGLUT2/DNPI operates as a vesicular glutamate transporter

In the following, we used several different and complementary approaches to evaluate the working hypothesis that VGLUT2/

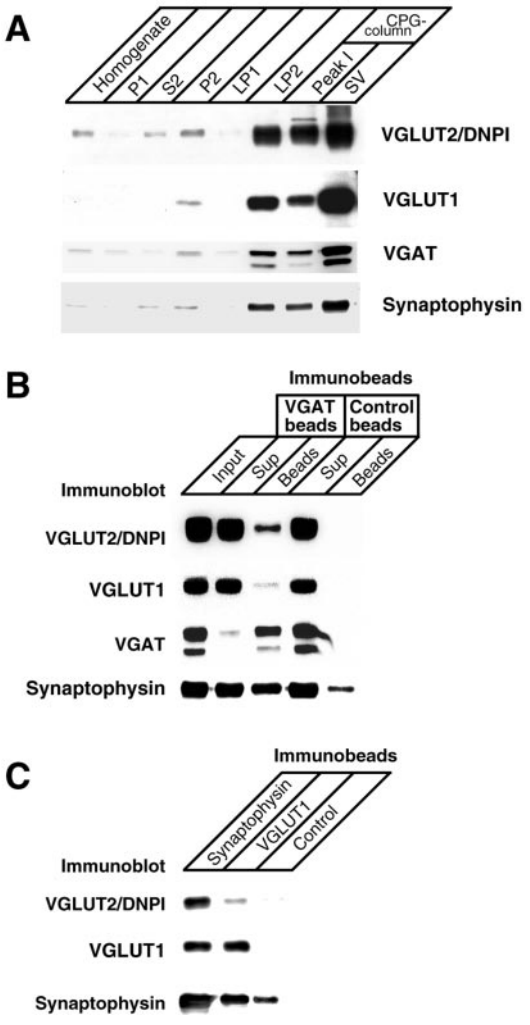

Figure 2. VGLUT2/DNPI is expressed on a synaptic vesicle population distinct from VGLUT1-containing vesicles and VGAT-containing vesicles. *A*, VGLUT2/DNP1 copurifies with synaptic vesicles during subcellular fractionation. Fractions obtained during purification of synaptic vesicles were analyzed by immunoblotting for the presence of VGLUT2/DNPI. The protein copurifies with other synaptic vesicle markers, with the highest enrichment in the purified synaptic vesicle fraction. *B*, Immuno-isolation of VGAT-containing vesicles reveals that neither VGLUT1 nor VGLUT2/DNPI are present on GABAergic synaptic vesicles. *Input*, Starting fraction (enriched vesicle fraction LP2); *Sup*, unbound material remaining in the supernatant after incubation with immunobeads; *Beads*, immunobead fraction. All samples were normalized to the same volume with respect to the input material. *C*, Immuno-isolation of VGLUT1-containing vesicles shows that VGLUT1 and VGLUT2/DNPI reside on different vesicle populations. For comparison, immunoisolates obtained with beads coated with synaptophysin-specific antibodies (*Synaptophysin*) or with inactivated beads (*Control*) were analyzed in parallel. Note that vesicles immuno-isolated with VGLUT1 immunobeads contain the same amount of VGLUT1 but less synaptophysin than the synaptophysin immuno-isolates, suggesting that the binding capacity of the VGLUT1 beads is somewhat lower but that VGLUT1 is enriched.

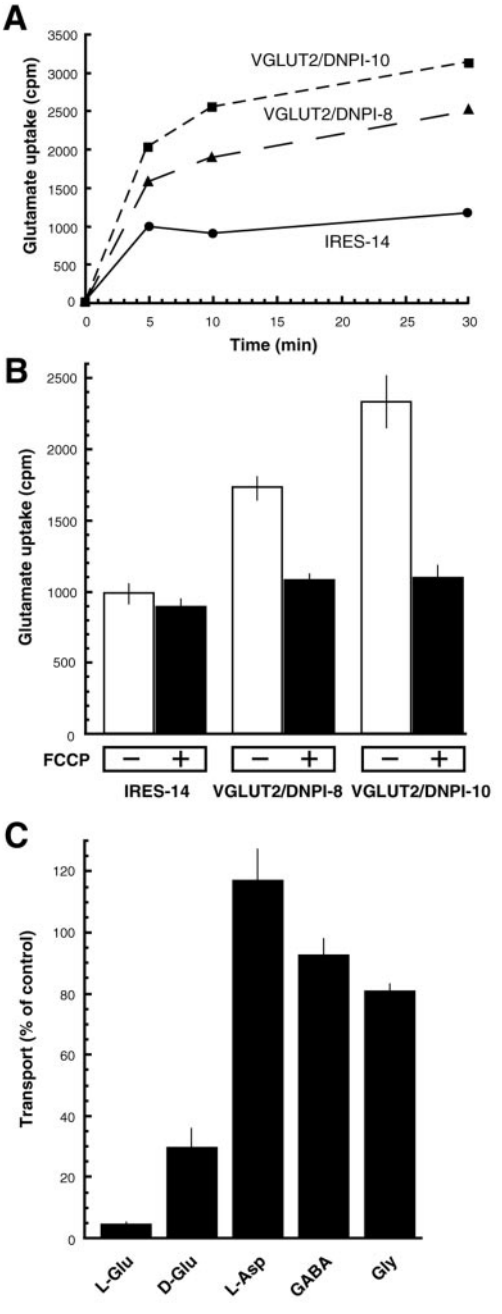

Figure 3. VGLUT2/DNPI functions as a vesicular glutamate transporter. *A*, Membrane fractions from VGLUT2/DNPI-expressing BON cell lines show ATP-dependent glutamate accumulation that is higher than that observed in membranes isolated in parallel from IRES-GFP transfected cells. Two independently selected cell lines (VGLUT2/DNPI-8 and VGLUT2/DNPI-10) stably expressing VGLUT2/DNPI were analyzed. *B*, Glutamate uptake by VGLUT2/DNPI is sensitive to the proton ionophore FCCP (final concentration of 46 μM), showing that uptake is driven by a proton electrochemical gradient. *C*, Substrate specificity of VGLUT2/DNPI. Uptake of [^{3}H]glutamate was competed for by 10 mM L-glutamate (*L-Glu*) and, to a somewhat lesser extent, by D-glutamate (*D-Glu*). In contrast, L-aspartate (*L-Asp*), GABA, and glycine (*Gly*) were unable to compete.

DNPI functions as a vesicular glutamate transporter. First, we established a cell line stably expressing VGLUT2/DNPI by transfecting a human serotonin-secreting cell line (BON cells) with cDNA encoding human VGLUT2/DNPI-IRES-GFP. Clones were selected based on GFP fluorescence. Expression of VGLUT2/DNPI in the GFP-positive cells was confirmed by immunostaining and/or immunoblotting using VGLUT2/DNPI-specific antisera (data not shown). After establishing two BON cell lines stably expressing VGLUT2/DNPI, membrane fractions were isolated and tested for ATP-dependent glutamate uptake using standard uptake conditions. As shown in Figure 3*A*, membranes from VGLUT2/DNPI-overexpressing BON cell lines

(VGLUT2/DNPI-8 and VGLUT2/DNPI-10) showed glutamate uptake activity that was significantly higher than that observed in membranes obtained from control cell lines (IRES-14). Incubation of membrane with the proton ionophore FCCP revealed that this difference is caused by a proton gradient-dependent process. Uptake activity in DNPI-expressing cells was reduced, whereas uptake activity in membranes of control cells remained unaffected. These data document that expression of VGLUT2/DNPI causes the expression of a glutamate uptake activity that is dependent on a transmembrane electrochemical proton gradient (Fig. 3*B*). Glutamate uptake mediated by VGLUT2/DNPI is specific for glutamate because the incorporation of [^{3}H]glutamate was competed for only by unlabeled L- and D-glutamate, whereas additions of other amino acid neurotransmitters were ineffective (Fig. 3*C*). In particular, no competition was observed with L-aspartate, showing that VGLUT2/DNPI, like VGLUT1, discriminates between glutamate and aspartate. These properties are identical to those described previously for glutamate uptake of synaptic vesicles (Naito and Ueda, 1985) and demonstrate, once again, that the substrate specificity of the vesicular transporters is different from the Na^+-dependent transport systems of the plasma membrane. Together, our findings show that VGLUT2/DNPI operates as a proton gradient-dependent glutamate transporter in internal membranes and exhibits properties very similar to those of VGLUT1 (Bellocchio et al., 2000; Takamori et al., 2000b).

To confirm the function of VGLUT2/DNPI with an independent approach, we examined whether neurons expressing VGLUT2/DNPI show synaptic release of glutamate during stimulation. We have shown previously that expression of VGLUT1 in GABAergic neurons results in the corelease of GABA and glutamate from the same cell, documenting that no other components are required to define a glutamatergic phenotype (Takamori et al., 2000b). Therefore, we used the same strategy to test whether VGLUT2/DNPI causes glutamate release if expressed in single autaptic GABAergic neurons. For this purpose, autaptic hippocampal neurons were infected with a Semliki Forest virus construct coexpressing VGLUT2/DNPI and GFP. GFP-positive GABAergic neurons were selected for whole-cell recordings. Autaptic responses were evoked by brief somatic depolarization (−70 to 0 mV for 1–2 msec) (Fig. 4*A*). All GABA-releasing cells exhibited a robust IPSC. In uninfected GABAergic cells, this current (3.39 ± 0.27 nA) was completely inhibited by the $GABA_A$ receptor antagonist bicuculline at saturating concentration (30 μM; $n = 5$) (Fig. 4*A*, *bottom trace*). However, in VGLUT2/DNPI-expressing GABAergic cells, we observed a fast inward current component that was resistant to saturating concentrations of the bicuculline. This current component had an average amplitude of 547 ± 160 pA ($n = 6$) and was identified as a glutamate-mediated EPSC because it was blocked by the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[F]quinoxaline (NBQX) (Fig. 4*A*, *top trace*, *B*). The decay time of bicuculline-resistant current developed during VGLUT2/DNPI overexpression was much faster than that of total current and was similar to that of normal glutamatergic EPSC (Fig. 4*C*), further indicating that the remaining current was mediated by glutamate. These results confirm that VGLUT2/DNPI, like VGLUT1, functions as a vesicular glutamate transporter in neurons and document that expression of VGLUT2/DNPI suffices to make neurons store glutamate in synaptic vesicles and release it by exocytosis.

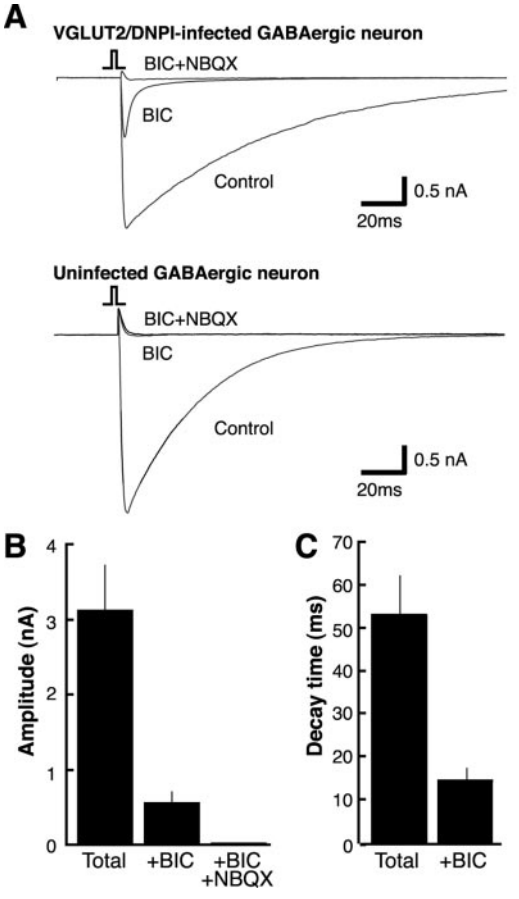

Figure 4. Expression of VGLUT2/DNPI suffices to induce corelease of GABA and glutamate from hippocampal autaptic GABAergic neurons. *A*, Hippocampal autaptic neurons were infected by Semliki Forest virus encoding human VGLUT2/DNPI-IRES-GFP. Only single, isolated neurons that exhibited green fluorescence were used for whole-cell patch recording. In VGLUT2/DNPI-infected GABAergic neurons, a postsynaptic current component resistant to 30 μM bicuculline was observed. These remaining currents were inhibited by 30 μM NBQX, suggesting that those currents were mediated by glutamate release. *B*, Mean postsynaptic currents observed from VGLUT2/DNPI-infected GABAergic neurons. *C*, Decay time of the bicuculline (*BIC*)-resistant current component compared with total postsynaptic currents.

DISCUSSION

In this study, we have shown that VGLUT2/DNPI is not only structurally similar to VGLUT1 but also operates as a vesicular glutamate transporter with properties very similar to VGLUT1. Both the subcellular localization and the functional properties strongly suggest that vesicular glutamate uptake is the principal function of these proteins. However, it remains to be established how the previously described activity as Na^+-dependent phosphate transporters can be integrated into the emerging picture (Otis, 2001). As discussed previously (Takamori et al., 2000b), it cannot be excluded that the transporter, when incorporated into the plasma membrane during exocytosis, mediates transport of inorganic phosphate. Furthermore, it remains to be established whether there is a link between phosphate and glutamate transport. For instance, it is possible that phosphate is exchanged for glutamate, at least under certain conditions, which would allow for charge neutrality during glutamate transport. To answer these questions is not easy, because a biochemical characterization of the transport activities is difficult as a result of the vesicular

localization of these transporters and their dependence on the membrane potential component of the proton gradient.

What is the difference between VGLUT1 and VGLUT2/DNPI? The regional expression patterns of VGLUT2/DNPI are different from that of VGLUT1. VGLUT2/DNPI is abundant in the medulla oblongata, thalamus, substantia nigra, and the spinal cord, whereas expression is weak or missing in other areas such as amygdala, the hippocampus, and cerebellum. This expression pattern nicely complements the expression of VGLUT1 that is highest in the cerebellum, the cerebral cortex, the amygdala, and the hippocampus, weak in the substantia nigra and the medulla, and absent from thalamus and spinal cord (Aihara et al., 2000; Hisano et al., 2000). Furthermore, our data show that synaptic vesicles contain predominantly, or even exclusively, either VGLUT1 or VGLUT2. (Fig. 2). It should be noted, however, that in recent studies VGLUT1 and VGLUT2/DNPI have been found to colocalize in some axon terminals (Sakata-Haga et al., 2001). However, the significance of colocalization of two isoforms of vesicular glutamate transporters at the same glutamatergic terminals is not yet clear. Interestingly, VGLUT2/DNPI was also found at some neurons that have been characterized as peptide-secreting neurons, such as corticotropin-releasing hormone (CRH) releasing neurons (CRHergic), orexin-releasing neurons (orexinergic), and melanin-concentrating hormone (MCH)-releasing neurons (MCHergic) in hypothalamus (Sakata-Haga et al., 2001). Thus, these well characterized peptide-secreting neurons might corelease glutamate from the same axon terminals, lending support to the idea that every peptidergic neuron also stores and releases a nonpeptide (classical) neurotransmitter.

So far, we have not found any functional difference between the two isoforms of vesicular glutamate transporters. Differences in substrate specificity and in inhibitor profiles were reported previously for the two isoforms of the vesicular monoamine transporter (Masson et al., 1999). However, it should be noted that much less is known about the transport mechanisms and the pharmacology of vesicular glutamate transporters, again primarily because of experimental problems. Therefore, additional studies may uncover subtle differences that may have an impact on glutamate-mediated neurotransmission, e.g., by changing the refilling mode of glutamate into synaptic vesicles and thus altering quantal size of glutamate.

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