Identification of the Differentiation-Associated Na⁺/P_i Transporter as a Novel Vesicular Glutamate Transporter Expressed in a Distinct Set of Glutamatergic Synapses

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Glutamate transport into synaptic vesicles is a prerequisite for its regulated neurosecretion. Here we functionally identify a second isoform of the vesicular glutamate transporter (VGLUT2) that was previously identified as a plasma membrane Na⁺-dependent inorganic phosphate transporter (differentiation-associated Na⁺/P_i transporter). Studies using intracellular vesicles from transiently transfected PC12 cells indicate that uptake by VGLUT2 is highly selective for glutamate, is H⁺ dependent, and requires Cl[−] ion. Both the vesicular membrane potential ($\Delta\psi$) and the proton gradient (Δ pH) are important driving forces for vesicular glutamate accumulation under physiological Cl[−] concentrations. Using an

antibody specific for VGLUT2, we also find that this protein is enriched on synaptic vesicles and selective for a distinct class of glutamatergic nerve terminals. The pathway-specific, complementary expression of two different vesicular glutamate transporters suggests functional diversity in the regulation of vesicular release at excitatory synapses. Together, the two isoforms may account for the uptake of glutamate by synaptic vesicles from all central glutamatergic neurons.

Key words: DNPI; BNPI; VGLUT1; VGLUT2; synaptic vesicle; excitatory synapse; vesicular glutamate transporter; glutamate release; chloride ion; synaptic plasticity; release probability

Before their release by exocytosis from the presynaptic nerve terminal, classical neurotransmitters are transported into synaptic vesicles (Jahn and Südhof, 1993). Within the last decade the proteins responsible for transporting biogenic amines, acetylcholine, and GABA/glycine into synaptic vesicles have been molecularly defined, and their distribution in the CNS has been characterized (Masson et al., 1999; Gasnier, 2000; Weihe and Eiden, 2000). Recently, a protein responsible for vesicular glutamate uptake (VGLUT1) has also been functionally identified (Bellocchio et al., 2000; Takamori et al., 2000) and is expressed in a subpopulation of glutamatergic neurons (Ni et al., 1995; Bellocchio et al., 1998).

Glutamate, the major excitatory neurotransmitter in the mammalian CNS, is selectively accumulated in synaptic vesicles where its concentration reaches ~60 mM, or approximately five to six times the cytoplasmic concentration (Storm-Mathisen et al., 1983; Burger et al., 1989). In contrast to Na⁺-dependent plasma membrane glutamate transporters, this vesicular glutamate uptake system displays low substrate affinity (~1 mM), is specific for L-glutamate, and is stimulated by physiologically relevant concentrations of Cl[−] ion (Disbrow et al., 1982; Naito and Ueda, 1983,

1985). To date there is no consensus in the literature regarding the role of Cl[−] ion or whether uptake of glutamate into synaptic vesicles is driven solely by the electric potential ($\Delta\psi$) or by both the $\Delta\psi$ and Δ pH components of the transmembrane electrochemical H⁺ gradient $\Delta\mu_{H^+}$ (Özkan and Ueda, 1998).

Identification of the brain-specific neuronal phosphate transporter (BNPI) as VGLUT1 was achieved when this protein was overexpressed in mammalian cell lines and found to mediate the transport of glutamate into isolated intracellular vesicles (Bellocchio et al., 2000; Takamori et al., 2000). Originally, BNPI was characterized as a type I phosphate ion transporter (Na⁺/P_i) on the basis of its weak (30%) homology with Na⁺/P_i-1 (Werner et al., 1991) and the ability of *Xenopus* oocytes expressing BNPI to accumulate radiolabeled ³²P_i (Ni et al., 1994). Recently a homologous protein was cloned (82% identity) and named DNPI for differentiation-associated Na⁺/P_i transporter (Aihara et al., 2000). The mRNA encoding DNPI displays a complementary distribution to VGLUT1, and when expressed in oocytes it also stimulates, albeit weakly, Na⁺-dependent ³²P_i transport (Aihara et al., 2000; Hisano et al., 2000).

To test the hypothesis that DNPI, like BNPI/VGLUT1, can function as a vesicular glutamate transporter, we developed a transient expression assay in PC12 cells in which nearly 100% of the cells express high levels of this protein. The ATP-dependent transport and accumulation of glutamate into intracellular vesicles by VGLUT2 revealed an obligatory role for Cl[−] and the ability to transport glutamate by either component of $\Delta\mu_{H^+}$. We raised antibodies against both VGLUT isoforms and found that excitatory synapses in the main sensory-motor and limbic-autonomic pathways in the brain and spinal cord exhibited marked differences and often mutual exclusivity in the distribution, density, and intensity of VGLUT1 and VGLUT2 puncta. In general, VGLUT1 is associated with neuronal pathways that

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exhibit activity-dependent potentiation, whereas VGLUT2 is expressed in sensory and autonomic pathways that display high-fidelity neurotransmission.

MATERIALS AND METHODS

Cloning of DNPI and plasmid construction. The cDNAs corresponding to the open reading frame of rat VGLUT2/DNPI and VGLUT1/BNPI were amplified by PCR from a cDNA library that was constructed from rat cerebellar granule cell cultures. The following primers for DNPI (5'-CGGGATCCCCTGGTAAGGCTGGACACGAGTCTTTACAAG and 5'-GCACCTTGATGGGACTCTACGGTCTGTTTGAATTC) and VGLUT1/BNPI (5'-GGAATTCCCGTGGGCACAGCCACCATGGAGTTCCGGCAG and 5'-GCTCTAGAGCCACCATGGGAGGCA-CGTGGTCAGTAGTC) were engineered to contain *Bam*HI and *Eco*RI restriction sites (for DNPI) or *Eco*RI and *Xba*I sites (for VGLUT1/BNPI) to facilitate subcloning into the mammalian expression vector pcDNA3.1 (Invitrogen). First, the cDNAs were purified and subcloned into pUC18, and overlapping fragments were sequenced in both directions with the Thermo Sequenase cycle sequencing kit (Amersham Biosciences) according to the manufacturer's instructions. Once the sequences were verified, they were subcloned into pcDNA3.1 for functional studies. The functional *lacZ* gene was excised from pCH110 at *Hind*III–*Bam*HI sites and subcloned into the mammalian expression vector pRCMV to monitor transfection efficiency. The recombinant vaccinia virus was prepared as described (Fuerst et al., 1986).

Preparation of glutathione S-transferase fusion proteins. For production of polyclonal antibodies, sequences corresponding to the coding region for the entire C-terminal hydrophilic portions of VGLUT2/DNPI (amino acids 501–582) and VGLUT1/BNPI (amino acids 493–560) were amplified by PCR from the respective cDNAs. The following primers for VGLUT2/DNPI (5'-CGGGATCCATTCATGAAGATGAACCTGGATGAAGAA and 5'-GGAATTCTTATGAATAATCATCTCCGGTCTTATAG) and VGLUT1/BNPI (5'-CGGGATCCGTTGGCCACGACAGCTGGCTGGCAGT and 5'-GGAATTCTCAGTAGTCCCGGACAGGGGGTGG) were designed to contain *Bam*HI and *Eco*RI sites that facilitated cloning into the bacterial expression vector pGEX-KT. To produce the fusion protein, the recombinant plasmids were transfected into BL21 cells, and protein expression in *Escherichia coli* was induced with isopropyl β -D-thiogalactoside for 4 hr at room temperature. The preparations were sonicated, and the recombinant glutathione S-transferase (GST) fusion proteins were isolated by chromatography using a bulk glutathione S-transferase purification module (Amersham Biosciences).

Polyclonal antibody production. New Zealand White rabbits were immunized with the GST fusion proteins. Rabbits were initially injected subcutaneously with 500 μ l of a 2 mg/ml solution of the fusion protein emulsified in Freund's complete adjuvant, and subsequent boosters with the same amount of protein were with incomplete adjuvant. Blood was obtained 14 d after each boost, serum was prepared, and 0.1% sodium azide was added as preservative.

Immunohistochemistry. Rats were anesthetized with ketamine/xylazine and transcardially perfused with PBS containing procaine-HCl (5 gm/l) followed by Bouin Hollande fixative as described (Nohr et al., 1999; Stumm et al., 2001). The perfused brains were removed, dissected into anterior, middle, and posterior parts, and post-fixed for 24–48 hr in Bouin Hollande fixative. After dehydration in a graded series of 2-propanol solutions, tissues were embedded in Paraplast Plus (Merck, Darmstadt, Germany). Deparaffinized serial sections were subjected to antigen retrieval by heating at 92–95°C for 15 min in 0.01 M citrate buffer, pH 6. Nonspecific binding sites were blocked with 5% bovine serum albumin in PBS followed by an avidin–biotin blocking step (avidin–biotin blocking kit, Boehringer Ingelheim, Heidelberg, Germany). For single peroxidase immunostaining, adjacent sections were alternately incubated with the primary polyclonal rabbit antisera raised against VGLUT1 (diluted 1:4000) and VGLUT2 (diluted 1:8000) overnight at 18°C followed by an additional incubation for 2 hr at 37°C. A polyclonal sheep antiserum against tyrosine hydroxylase (diluted 1:1000; Chemicon) was applied to identify catecholaminergic cell groups and terminals. After several washes in distilled H₂O followed by rinsing in 50 mM PBS, species-specific biotinylated secondary antibodies (Dianova, Hamburg, Germany) were applied for 45 min at 37°C. After another series of washes, sections were incubated for 30 min with the ABC reagents (Vectastain Elite ABC Kit, Boehringer Ingelheim). Immunoreactions were visualized with 3',3'-diaminobenzidine (Sigma, Deisenhofen, Ger-

many) enhanced by the addition of 0.08% ammonium nickel sulfate (Fluka, Buchs, Switzerland), which resulted in a dark blue staining. The specificity of VGLUT1 and VGLUT2 immunostaining was tested by preadsorption with the respective homologous recombinant fusion proteins (1 μ M).

Confocal laser scanning. Confocal laser scanning double-immunofluorescence analysis was performed as described previously (Stumm et al., 2001). Sections were incubated overnight at room temperature with a mixture of the polyclonal rabbit VGLUT1 (1:300) or VGLUT2 (1:600) and a monoclonal mouse antibody against the synaptic vesicles marker synaptophysin (Clone SY 38; Roche Molecular Biochemicals; diluted 2 μ g/ml) or a monoclonal mouse antibody against microtubule-associated protein-2 (MAP-2), an established marker of neuronal dendrites (MAB3418; Chemicon International, Temecula, CA; diluted 25 μ g/ml). Immunoreactivities were visualized with indocarbocyanine-conjugated species-specific secondary antibodies diluted 1:200 and applied for 45 min at 37°C, resulting in a red-orange fluorescence labeling, or with biotinylated IgG (Dianova) diluted 1:200, applied for 45 min at 37°C, followed by incubation with Alexis 488-conjugated streptavidin (MoBiTec, Göttingen, Germany) for 2 hr at 37°C, resulting in a green fluorescence. Sections were analyzed with the Olympus Fluoview confocal laser scanning microscope (Olympus Optical, Hamburg, Germany) and documented as false color confocal images.

Synaptosome preparation and fractionation. The subcellular fractionation of a crude synaptosomal preparation from rat brain was performed as described by Huttner et al. (1983) with minor modifications. Two rat brains were homogenized in 30 ml of homogenization buffer (0.32 M sucrose, 10 mM HEPES-NaOH, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/l pepstatin, 5 mg/l leupeptin, and 5 mg/l aprotinin) using a glass–Teflon homogenizer (12 strokes, 900 rpm). The homogenate was centrifuged 10 min at 800 $\times$ g, the pellet (P1) was saved, and the supernatant was centrifuged again for 15 min at 9200 $\times$ g. The pellet was resuspended in 20 ml of homogenization buffer and centrifuged again at 10,200 $\times$ g to yield a washed synaptosomal pellet (P2). The supernatants of the last two spins were pooled (S2). The synaptosomes in P2 were resuspended in homogenization buffer (final volume 5 ml), hypo-osmotically lysed by addition of 45 ml of ice-cold 5 mM HEPES-NaOH, pH 7.4, containing protease inhibitors, and homogenized with a glass–Teflon homogenizer (10 strokes; 900 rpm) followed by rocking at 4°C for 15 min. The lysed synaptosomes were centrifuged for 20 min at 25,000 $\times$ g, and the resulting LP1 pellet was saved. The supernatant was centrifuged again for 1 hr at 50,000 rpm in a Ti50.4 rotor to obtain LP2 pellets and the LS2 supernatant. The pellets were resuspended in a total of 5 ml of 25 mM sucrose and layered on top of a linear continuous sucrose gradient made from 16 ml of 50 mM sucrose and 15 ml of 800 mM sucrose. Centrifugation was performed for 150 min in an SW 28 rotor at 25,000 rpm, and the broad turbid band in the 200–500 mM sucrose region containing synaptic vesicles was collected and centrifuged in a 70 Ti rotor for 2 hr at 40,000 rpm. The microsomal pellet at the bottom of the sucrose gradient was saved. The protein concentration of all fractions was determined with the Bradford assay (Bio-Rad) after dilution (5 $\times$) in PBS containing 0.1% SDS using bovine serum albumin as the standard.

Synaptic vesicle preparation. Synaptic vesicles were also purified from rat forebrain using a modified procedure of Jahn and coworkers (Hell et al., 1988). Briefly, 15 gm of rat brain was frozen and pulverized in liquid nitrogen to a fine powder. The powder was resuspended in 100 ml of sucrose buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM PMSF, 6 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 5 μ g/ml pepstatin, and homogenized in a tight-fitting glass–Teflon homogenizer (nine strokes, 900 rpm). The homogenate was centrifuged for 10 min at 47,000 $\times$ g followed by centrifugation at 120,000 $\times$ g for 2 hr. The supernatant (20 ml) was layered onto a cushion (5 ml) of 0.6 M sucrose, 10 mM Tris-HCl, pH 7.4, and centrifuged at 260,000 $\times$ g for 2 hr. The pellet was resuspended in 3 ml of sucrose buffer (without inhibitors) and cleared by centrifugation at 27,000 $\times$ g for 10 min. Synaptic vesicle preparations were aliquoted and stored at –70°C without loss of activity.

Western analysis. Samples containing 2–10 μ g of protein were resuspended in sample buffer containing 62 mM Tris-HCl, pH 6.8, 1 mM EDTA, 10% glycerol, 5% SDS, and 50 mM dithiothreitol, fractionated by SDS-PAGE using an 8% polyacrylamide gel, and electrophoretically transferred onto nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). After a 1 hr preincubation in TBS (0.2 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk, the blots were incubated for 3 hr at room temperature with primary antibodies in TBS–1% bovine

serum albumin. BNPI/VGLUT1 (1:1000), DNPI/VGLUT2 (1:1000), or synaptophysin (1:5000; Sigma) was detected using horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (Sigma) and enhanced chemiluminescent reagents (Amersham Biosciences) followed by exposure to film.

Transient infection/transfection of PC12 cells. Wild-type rat PC12 cells (gift of John A. Wagner, Cornell University Medical College) were maintained at 37°C in an atmosphere of 90% air, 10% CO₂ in DMEM containing 10% fetal bovine serum, 5% heat-inactivated horse serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and glutamine (4 mM). PC12 cells (8 × 10⁶) were plated in 10 cm² dishes precoated with polyornithine (4 μg/ml; Sigma). The following day the cells were rinsed with PBS and infected with a recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase at a multiplicity of infection of 20 for 2 hr (Fuerst et al., 1986). The medium was removed, and the cells were transfected with DMEM (2 ml) containing T7 promoter-bearing plasmid cDNA (2 μg/ml) and lipofectamine2000 (1:100) for 6 hr, and fresh medium (5 ml) containing 2.5% fetal bovine serum was added. After 16–18 hr the cells were harvested, and vesicle membranes were prepared.

β-Galactosidase staining assay. Transfection efficiency was generally monitored in a parallel 10 cm² dish of PC12 cells transfected with the *lacZ* gene using a histochemical stain for β-galactosidase (Sanes et al., 1986). The medium was removed, and the cells were fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 5 min at room temperature. After three successive washes in PBS (5 min each), 10 ml of a filtered (0.45 μm) solution containing 5 mM potassium ferri cyanide and 5 mM potassium ferro cyanide in PBS and X-gal (1 mg/ml) was added. The cells were then placed in a 37°C incubator for up to 1 hr.

Preparation of vesicle membranes from PC12 cells. Control and VGLUT2-expressing PC12 cells were collected, rinsed in PBS, and homogenized with a ball-bearing device (11 μm clearance) in ice-cold buffer containing 0.32 M sucrose, 10 mM HEPES, pH 7.4, 1 mM PMSF, 6 μg/ml leupeptin, 5 μg/ml aprotinin, and 5 μg/ml pepstatin. The resulting homogenates were cleared by successive centrifugation at 2000 × *g* for 10 min and 10,000 × *g* for 10 min to remove nuclei, mitochondria, and cell debris. The supernatant was sedimented by centrifugation at 200,000 × *g* for 45 min in a TLA100 rotor. The resulting membrane pellet was resuspended in 0.32 M sucrose, 10 mM HEPES, pH 7.4, containing protease inhibitors. Protein was measured by the Bradford assay (Bio-Rad) using bovine serum albumin as the standard.

Vesicular [³H]L-glutamate transport assay. For [³H]L-glutamate uptake assay, aliquots (100 μl) of membranes containing 100 μg of protein were mixed with uptake buffer (50 μl) containing 110 mM potassium tartrate, 20 mM HEPES, pH 7.4, in the presence and absence of various anions or inhibitors and incubated at 32°C for 2 min. After preincubation, a solution (50 μl) containing 20 mM Mg²⁺-ATP (neutralized with KOH to pH 7.4) and 200 μM glutamate containing 1 μM [³H]L-glutamate (42.9 Ci/mmol; DuPont NEN) in the presence and absence of various competitive inhibitors were added. The final concentration of MgATP was 5 mM. For kinetic analysis, vesicle preparations were incubated with 0.25 μM [³H]L-glutamate and increasing concentrations (0.05–6.4 mM) of unlabeled glutamate in the presence of 4 mM KCl, and uptake was terminated after 4 min. Membranes from mock-transfected PC12 cells and PC12 cells expressing DNPI/VGLUT2 were always analyzed in parallel to assess background uptake. The uptake reactions were stopped by placing tubes in ice water, and the samples were vacuum filtered through glass fiber filters (GF/F) and washed with 6 ml ice-cold uptake buffer containing 10 mM MgSO₄. Radioactivity bound to the filters was solubilized in 1 ml of 1% SDS followed by addition of 10 ml of EcoScint scintillation fluid and quantitated by liquid scintillation counting. Experiments were performed in duplicate or triplicate and repeated two to four times. *K_m* values were determined by nonlinear regression (Prism3, Graphpad Software).

RESULTS

DNPI is a novel glutamatergic synaptic vesicle marker

We raised antibodies to fusion proteins containing the cytoplasmic C termini of DNPI and its homolog VGLUT1 to compare the subcellular localization and cellular distribution of these proteins. The antisera recognized distinct broad bands (60 and 55 kDa) for DNPI and VGLUT1 on Western blots of synaptic vesicles purified from rat brain (Fig. 1*A*). When transiently expressed in PC12 cells, which do not express these proteins endogenously, we found

Figure 1. Antibody specificity and subcellular localization of VGLUT1 and VGLUT2 isoforms. *A*, Western blot strips of purified rat brain synaptic vesicles (Hell et al., 1988) were prepared and probed with rabbit polyclonal antisera raised against a GST fusion protein containing the C-terminal tails of rat VGLUT2 (DNPI) or VGLUT1 (BNPI). Broad bands are observed for VGLUT2 and VGLUT1 (– lanes) unless 10 μg/ml of the cognate fusion proteins are added (+ lanes). *B*, VGLUT2 and VGLUT1 are *N*-glycosylated. Rat brain synaptic vesicles (10 μg) were incubated for 1 hr at 37°C in the absence (–) or presence (+) of peptide-*N*-glycosidase F. A faster migrating species is observed after digestion. *C*, Fractionation of rat brain synaptosomes. The following rat brain fractions were analyzed for the presence of VGLUT2, VGLUT1, and the synaptic vesicle marker synaptophysin (*p38*): brain homogenate (*TOTAL*), low-speed pellet (*P1*), washed synaptosomes (*P2*), supernatant of the synaptosomal fraction (*S2*), low-speed pellet of the lysed synaptosomes (*LP1*), high-speed pellet of the lysed synaptosomes (*LP2*), synaptosomal cytosol (*LS2*), synaptic vesicles (*SV*) purified from *LP2* by continuous sucrose density gradient fractionation, and the microsomal fraction (*Microsome*) of lysed synaptosomes.

that the antisera raised against each isoform were completely specific (data not shown). Inclusion of GST fusion proteins used as immunogens abolished immunoreactivity on Western blots (Fig. 1*A*), which confirms the specificity of the antibodies. These reagents are thus suitable for mapping the expression of each isoform in the rat nervous system. Pretreatment of rat brain vesicle membranes with *N*-glycanase resulted in a shift in the electrophoretic mobility of both DNPI and VGLUT1 (Fig. 1*B*), indicating that both of these proteins are *N*-glycosylated. Putative *N*-glycosylation sites are located between the first two putative transmembrane helices similar to the vesicular amine transporters

Figure 2. Characterization of vesicular glutamate transport by VGLUT2 in transiently transfected PC12 cells. *A*, Transfection efficiency close to 100% and high expression levels are obtained as illustrated by the strong labeling visible after 30 min incubation with X-gal at 37°C in β -galactosidase-expressing PC12 cells. *B*, VGLUT2 is detected in transfected but not in mock-transfected PC12 cell homogenates. *C*, Time course of glutamate uptake in VGLUT2-transfected (●) and mock-transfected (○) PC12 cells. *D*, Saturation analysis of glutamate uptake by VGLUT2. VGLUT2-specific (mock-subtracted) uptake velocity reaches a plateau at ~2 mM substrate. *Inset*, Lineweaver-Burk analysis of VGLUT2 initial velocity determines a K_m of 0.8 mM and a V_{max} of 190 pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ protein. *E*, VGLUT2 is highly specific for glutamate. Transport of glutamate (50 μ M) was measured in 4 mM Cl $^-$ -containing medium in the absence (100%) or presence of various amino acids (10 mM), K $_2$ PO $_4$ (20 mM), or trypan blue (TB) (1 μ M). *F*, VGLUT2 activity is H $^+$ dependent. H $^+$ -ATPase inhibitors and dissipaters of the H $^+$ electrochemical gradient abolish transport. Vesicle preparations were preincubated for 2 min with 1 μ M bafilomycin A1 (bafilo), 200 μ M *N*-ethyl maleimide (NEM), 50 μ M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), or 20 mM KSCN (SCN). *G*, Anion specificity of VGLUT2 stimulation. Vesicle preparations were incubated in the absence or presence of the potassium salts of various inorganic anions (4 mM), chloride (Cl $^-$), bromide (Br $^-$), iodide (I $^-$), phosphate (PO $_4$ $^{3-}$), thiocyanate (SCN $^-$), and in the presence of Cl $^-$ plus 1 μ M DIDS or 4 mM SCN $^-$. *H*, Transport is dependent on $\Delta\psi$ but can be driven by ΔpH . Vesicle preparations were incubated in the absence (control) or presence of 1 μ M nigericin (+Nig), 1 μ M valinomycin (+Val), or both (+Nig, +Val), in a medium containing 4 or 40 mM Cl $^-$ and 5 mM Mg-ATP. Nonspecific transport (corresponding mock values) was subtracted.

(Varoqui and Erickson, 1997). To determine whether DNPI was enriched on synaptic vesicles, we fractionated synaptosomes prepared from rat cerebrum. Immunoblot analysis revealed that DNPI, like VGLUT1, followed a distribution identical to the one of synaptophysin, a well established marker for synaptic vesicles (Fig. 1C). The high homology of DNPI (82%) to the glutamate vesicular transporter VGLUT1 and its enrichment on synaptic vesicles suggested that, in addition to transporting P $_i$ across the plasma membrane (Aihara et al., 2000), DNPI may function as a vesicular transporter.

Functional identification of DNPI as a vesicular transporter for glutamate (VGLUT2)

To determine whether DNPI functions as a vesicular glutamate transporter, the cDNA was transiently expressed in PC12 cells using the vaccinia virus/bacteriophage T7 system. Generally, up to 95% of the cells were found to express high levels of exogenous protein using this method (Fig. 2A). When a population of light vesicle membranes was prepared and analyzed by Western blot, a

predominant band of the expected molecular weight for DNPI was observed in PC12 cells transfected with this cDNA and not in mock-transfected cells (Fig. 2B). Uptake of glutamate by these PC12 vesicles was measured in the presence of Mg $^{2+}$ -ATP (5 mM) and KCl (4 mM), conditions known to be optimal for glutamate transport by synaptic vesicles isolated from brain (Naito and Ueda, 1985). Glutamate uptake into vesicles from DNPI-expressing cells was generally three to four times greater than that seen with mock-transfected controls (Fig. 2C) and had properties similar to glutamate transport by vesicle membranes from stable cell lines expressing VGLUT1 (Bellocchio et al., 2000; Takamori et al., 2000). The initial rate of glutamate uptake (Fig. 2D) measured during the linear portion of the time course (4 min) was saturable with an apparent K_m of 0.8 mM and V_{max} of 190 pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ ($n = 2$). Transport mediated by DNPI was highly selective for glutamate because L-aspartate, L-glutamine, and GABA did not interfere with the uptake of glutamate when they were present at 10 mM (Fig. 2E). Uptake was signifi-

cantly reduced in the presence of 1 μM trypan blue, an inhibitor of vesicular glutamate uptake in synaptic vesicles (Roseth et al., 1998). Although 10 mM PO_4^{4-} did not appear to inhibit uptake by VGLUT1 (Bellocchio et al., 2000), uptake by VGLUT2 was reduced 50% in the presence of 20 mM PO_4^{4-} (Fig. 2E). Glutamate uptake by DNPI was dependent on exogenous ATP and was abolished by bafilomycin and NEM, which are specific inhibitors of the vacuolar-type H^+ -ATPase, and by the proton ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 50 μM), indicating that transport is dependent on the H^+ electrochemical gradient (Fig. 2F). These results provide compelling evidence that DNPI functions as a vesicular transporter that is highly specific for glutamate, and we propose to rename the protein VGLUT2.

ATP-dependent glutamate uptake into vesicles by VGLUT2 requires Cl^- ion

Low, physiologically relevant concentrations of Cl^- enhanced the VGLUT2-mediated uptake of glutamate by $\sim 400\%$ (Fig. 2G). The minimal Cl^- concentration that was required for maximum glutamate uptake was 2–4 mM, but a similar stimulation of glutamate uptake was also observed at 16 and 20 mM Cl^- ion. Uptake of glutamate in buffered KCl (150 mM) was not observed (data not shown). The addition of 4 mM Cl^- or Br^- anions increased glutamate uptake into VGLUT2-expressing vesicles, whereas I^- was less effective. Neither 4 mM PO_4^{4-} nor SCN^- ions had any effect on uptake. These experiments indicate that glutamate uptake by VGLUT2 is differentially regulated by anions.

The activation of glutamate uptake by Cl^- or Br^- ions has been attributed to their direct interaction with the cytoplasmic face of the transporter via an anion-binding site (Harterter and Jahn, 1993) or their acting on a vesicular anion channel to increase ΔpH (Xie et al., 1989; Tabb et al., 1992). Low concentrations of SCN^- (4 mM) partially antagonized the Cl^- stimulation of glutamate uptake, as did the anion transport blocker 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) when present at 1 μM (Fig. 2G). These results are consistent with the possibility that SCN^- and DIDS may compete with Cl^- ion for binding either at a vesicular anion channel or directly on the transporter. In the presence of higher concentrations of SCN^- (20 mM), which selectively dissipate $\Delta\psi$ (Johnson et al., 1981), the uptake of glutamate was abolished (Fig. 2F).

VGLUT2-mediated uptake into vesicles depends on both ΔpH and $\Delta\psi$

To further assess the dependence of glutamate transport on $\Delta\psi$ and ΔpH , we used a combination of ionophores that selectively dissipate the electrical or chemical components of the $\Delta\mu_{\text{H}^+}$ (Fig. 2H). Nigericin is an electroneutral K^+/H^+ exchanger that selectively dissipates ΔpH , whereas valinomycin, a K^+ -ionophore, selectively dissipates $\Delta\psi$. Glutamate transport was assayed in the presence of 4 and 40 mM Cl^- , conditions in which transmembrane $\Delta\psi$ predominates or both $\Delta\psi$ and ΔpH gradients exist (Tabb et al., 1992; Moriyama and Yamamoto, 1995). Uptake of glutamate in the presence of 40 mM Cl^- is reduced $\sim 25\%$ from that observed at 4 mM Cl^- . A biphasic dependence of vesicular glutamate uptake on Cl^- has also been reported for VGLUT1 (Bellocchio et al., 2000) and observed in isolated brain vesicles (Naito and Ueda, 1985). Valinomycin (10 μM) reduces glutamate uptake in both Cl^- conditions by $>80\%$, indicating that $\Delta\psi$ is a primary driving force for accumulation. The uptake of glutamate by VGLUT2 at 4 mM Cl^- is modestly inhibited (20%) in the

Figure 3. Confocal laser scanning double-immunofluorescence microscopy for VGLUT2 or VGLUT1 and the synaptic vesicle nerve terminal marker synaptophysin or the dendritic marker MAP-2. False color micrographs of confocal images from double immunofluorescence for VGLUT2 or VGLUT1 (green) and synaptophysin and microtubule-associated protein-2 (MAP2) (red) in the spinal dorsal horn. VGLUT2 and VGLUT1 staining coincide with staining for synaptophysin and are absent from neuronal dendrites. Note the presence of VGLUT2 staining in perisomatic and peridendritic synapses. Scale bar, 10 μm .

presence of 1 μM nigericin, an effect similar to that reported for VGLUT1 (Takamori et al., 2000), indicating that ΔpH is not a significant driving force under these conditions. However, uptake observed with VGLUT2 in 40 mM Cl^- is abolished by nigericin, indicating that H^+ may be directly involved in glutamate countertransport, as suggested by Ueda and colleagues (Shioi and Ueda, 1990; Tabb et al., 1992).

Differential distribution pattern of VGLUT2 and VGLUT1

To compare the distribution of VGLUT2 in relation to that of VGLUT1 throughout the brain and spinal cord, we used light microscopic immunohistochemistry on pairs of adjacent sections alternately stained for VGLUT2 and VGLUT1. Specific staining for VGLUT2 and VGLUT1 was found to be restricted to synaptic puncta in brain and spinal cord areas known to receive glutamatergic input. As a rule, neuronal perikarya and dendrites remained unstained. With double-immunofluorescence and confocal laser scanning microscopy it could be shown that VGLUT1 and VGLUT2 immunoreactivity (ir) coincided with staining for the synaptic marker synaptophysin and was strictly segregated from the staining for the neuropil and the neurodendritic marker MAP2 (Fig. 3). The specificity of the VGLUT1 and VGLUT2-ir was assessed by preabsorption of the antisera with the homologous recombinant fusion protein. This is exemplarily shown for the spinal cord (Fig. 4). Furthermore, immunostaining for the two vesicular glutamate transporters was distinct from staining of other classical neurotransmitter systems, including

Figure 4. The distribution and assessment of the specificity of VGLUT2 and VGLUT1-ir in lumbar spinal cord. Immunoreactions in *A* and *C* are fully preabsorbed with the homologous recombinant fusion protein (*B*, *D*). Punctate immunostaining for VGLUT2 is present in the superficial dorsal horn (*A*, arrowheads mark lamina 1 and substantia gelatinosa) where VGLUT1-ir is minimal (*C*, arrowheads). Note accumulation of strongly positive punctate VGLUT1-ir in the deep dorsal horn where VGLUT2-ir is comparatively low. VGLUT2-ir is present in the lateral spinal nucleus (*LSN*) (*A*, arrows) where VGLUT1-ir is minimal (*C*, arrows). VGLUT1 immunostaining is low to moderate in the lateral ventral horn and sparse in the medial ventral horn. Fine punctate VGLUT2 staining is dense and abundant in the ventral horn (*VH*). Scale bar, 500 μ m.

cholinergic synapses, that were visualized by double-staining for the cholinergic marker VACHT, the vesicular acetylcholine transporter, catecholaminergic synapses using the marker tyrosine hydroxylase, and GABAergic/glycinergic synapses using the vesicular inhibitory amino acid transporter (data not shown). Thus, the specificity of VGLUT1 and VGLUT2 staining for glutamatergic synapses is unambiguous.

We revealed mostly segregated and complementary distribution of VGLUT1 and VGLUT2 synapses in the different telencephalic and diencephalic nuclei and in the spinal cord that is in accordance with recent, less extensive analyses (Fujiyama et al., 2001; Sakata-Haga et al., 2001).

Spinal cord sensory and motor pathways

Pain processing areas of the substantia gelatinosa and the lateral spinal nucleus in the dorsal lumbar spinal cord contained numerous VGLUT2 positive puncta but only extremely sparse VGLUT1 puncta (Fig. 4). An accumulation of VGLUT1 puncta was observed in deep dorsal horn nucleus proprius where cutaneous and deep mechanoreceptive primary afferents terminate. Here, VGLUT2 puncta were relatively sparse (Fig. 4). VGLUT2

but not VGLUT1 was quite abundant in lamina X around the central canal. VGLUT2 puncta were present around preganglionic sympathetic and parasympathetic visceromotor neurons of the intermediolateral thoracolumbar and intermediomedial sacral cell columns (data not shown). Throughout the ventral horn, fine VGLUT2 puncta were abundant (Fig. 4). In contrast, VGLUT1 puncta in the ventral horn were less dense and larger in size than VGLUT2 puncta and preferentially distributed to the lateral ventral horn where they appeared to target motoneuron perikarya and dendrites.

Cerebrocortical and hippocampal pathways

In the neocortex of frontal, parietal, temporal, and occipital lobes, VGLUT2 staining was relatively low, with a marked preference for the granular layer of lamina IV, as demonstrated for the parietal cortex (Fig. 5). The preferential sensory input of VGLUT2 synapses to layer IV, in conjunction with abundant VGLUT2 mRNA expression in thalamic nuclei known to project to the neocortex (Hisano et al., 2000), indicates that cortical VGLUT2 synapses originate mainly from glutamatergic thalamocortical projection neurons. In contrast, VGLUT1 puncta were very densely distributed throughout neocortical layers I–VI and were lower in lamina IV where VGLUT2 was concentrated. The vast majority of cortical VGLUT1 terminals are likely to originate from corticocortical projections of glutamatergic pyramidal neurons shown previously to express VGLUT1 mRNA (Ni et al., 1994, 1995). The distinct complementary relationship between VGLUT2 and VGLUT1 in the superficial lamina I and in lamina II–IV of the retrosplenial cortex also suggests layer- and pathway-specific diversification of vesicular glutamate storage in the cerebral cortex (Fig. 5). In the piriform cortex, VGLUT2 was restricted to fine puncta of the pyramidal cell layer, whereas VGLUT1 puncta were abundant throughout all layers except the pyramidal layer (Fig. 6*A,C,E,G*). Throughout the olfactory tubercle dendritic region, VGLUT1 synaptic input prevailed, whereas sparse VGLUT2 puncta were distributed around cell bodies (Fig. 6*A,E*). VGLUT1 was abundant in the peripheral dendritic area of the islands of Calleja, whereas VGLUT2 dominated in the center bearing the perikarya (Fig. 6*D,H*).

A striking pattern of mutually exclusive and complementary distribution of the two vesicular glutamate transporters was obvious in the dentate gyrus and the Ammon's horn. VGLUT2 synapses show preference to the pyramidal layers of CA1–CA3 and the granular layer of the dentate gyrus. The origin of these synapses is presently unknown, but because VGLUT2 mRNA has also been reported in these cell body layers (Hisano et al., 2000) they may be excitatory interneurons. VGLUT2 synapses are virtually absent from entorhinal glutamatergic inputs through the perforant path to the molecular layer in the dentate gyrus, from glutamatergic mossy fiber terminals in stratum lucidum of CA3, and from synapses of glutamatergic Schaffer collaterals in the stratum oriens and radiatum of CA1 and CA3. Here, VGLUT1 protein is extremely abundant (Fig. 5).

Basal forebrain systems

Basal forebrain regions receiving both VGLUT1 and VGLUT2 synaptic input showed microterritorial differences in their distribution and density patterns that were grossly complementary. For example, in the nucleus accumbens, VGLUT1 terminals dominated in the core where VGLUT2 innervation was minimal. Within the nucleus accumbens shell, mutually exclusive

Figure 5. The distribution of VGLUT2 and VGLUT1 in the forebrain. *A, H*, Punctate VGLUT2-ir is relatively abundant throughout the hypothalamus and thalamus where VGLUT1 is restricted to the hypothalamic ventral premammillary nucleus (*PMV*) and to parts of the thalamic nuclei including the lateral posterior thalamic nucleus (*LP*), dorsal lateral geniculate nucleus (*DLG*), and ventral posteromedial thalamic nucleus (*VPM*). *OPT*, Olivary pretectal nucleus; *APT*, dorsal anterior pretectal nucleus; *PrC*, precommissural nucleus. VGLUT2 staining is moderate in a band of the neocortex comprising lamina IV, weak in a neocortical band comprising lamina VI, and minimal in the other neocortical layers. Intense punctate VGLUT1-ir is abundant throughout the cortex including the piriform cortex (*Pir*) and somewhat less strong in the neocortical band of lamina IV where moderate VGLUT2 staining accumulates. Note mutual exclusion of VGLUT1 and VGLUT2 staining in the layers of the retrosplenial granular cortex (*A, H, RSG*) shown at high magnification in *E* and *L*. High magnifications (*G, N*) from lamina IV in *F* and *M* demonstrate different densities of VGLUT1- and VGLUT2-ir puncta between immunonegative neuronal cell bodies and processes. Sparse VGLUT2-ir puncta are mostly confined to the granular layer (*g*) of the dentate gyrus (*DG*) and pyramidal layer (*p*) of the fields CA1, CA2, and CA3 of the hippocampus. Dense VGLUT1-ir puncta are abundant throughout the hippocampus with the exception of the granule (*g*) and pyramidal (*p*) cell layers. Areas in rectangles in *A* and corresponding areas on adjacent section in *H* are shown at high magnification in *B–D* and *I–K*, respectively. Note differential distribution and density of VGLUT1-ir and VGLUT2-ir in the oriens layer (*o*), pyramidal layer (*p*), stratum radiatum (*r*), and stratum lacunosum moleculare (*l*) of CA1 (*B, I*) and CA3 (*C, J*) and in the molecular (*m*), granular (*g*), and polymorphic (*p*) layer of the dentate gyrus (*DG*) (*D, K*). Note some overlap but differential density and intensity of immunostaining for VGLUT1-ir and VGLUT2-ir puncta in the posterior basomedial amygdaloid nucleus (*BMP*), in the lateral amygdaloid nucleus (*La*), and in the cortical amygdaloid nucleus (*Co*) as well as in the adjacent dorsal endopiriform nucleus (*DEn*). Note that white matter and fiber tracts are VGLUT1 and VGLUT2 negative. *pc*, Posterior commissure; *f*, fornix; *fr*, fasciculus retroflexus; *mt*, mammillothalamic tract. Scale bars: *A, E*, 1.5 mm; *B–D*, 50 μ m; *F–H*, 50 μ m; *I, J*, 100 μ m; *K, L*, 50 μ m; *M, N*, 12.5 μ m.

VGLUT2- and VGLUT1-dominated areas were obvious. The cholinergic region of the vertical limb of the diagonal band received a selective VGLUT2 synaptic input. In contrast, the lateral septum received both VGLUT1 and VGLUT2 synaptic input in a subregion-specific complementary manner (Figs. 5, 6). Also in the amygdaloid complex, mutually exclusive density gradients between VGLUT1 and VGLUT2 synaptic puncta were observed. Cortical and subcortical amygdaloid regions received a predominant VGLUT1 input. In contrast, medial regions such as the basomedial amygdaloid nucleus obtained a predominant VGLUT2 input (Fig. 5*A, H*). Thus, the two VGLUT isoforms could be specifically involved in disturbed interactions between glutamatergic and dopaminergic neurotransmission suggested to be involved in psychiatric illness, especially schizophrenia, and drug dependence (Carlsson et al., 2001; Floresco et al., 2001; Pulvirenti and Diana, 2001).

Extrapyramidal motor system of basal ganglia and mesencephalon

Throughout the caudate-putamen, fine VGLUT2- and VGLUT1-positive puncta were evenly distributed, with VGLUT1 puncta exhibiting a slightly higher density than VGLUT2 puncta (Fig. 6). Most of the demonstrated VGLUT1 synaptic input to the caudate-putamen is likely to originate from cortical pyramidal neurons expressing abundant VGLUT1 mRNA (Ni et al., 1994, 1995). VGLUT2 synaptic input is probably mainly derived from thalamostriatal projections. Glutamatergic projections of the subthalamic nucleus to the globus pallidus and the substantia nigra pars compacta (SNr) and pars reticulata (SNr) can now be classified as operating exclusively with VGLUT2 (Figs. 6, 9). In some neurons of the SNr, weak to moderate staining for VGLUT2 was seen in perikarya. This was one of the very few

Figure 6. The distribution of VGLUT2 and VGLUT1 in the forebrain. Marked differences are apparent in the distribution, density, and intensity of punctate VGLUT2-ir (*A–D*) and VGLUT1-ir (*E–H*) in the neocortex (lamina *IV*, *VI*), caudate putamen (*CPu*), globus pallidum (*GP*), piriform cortex (*Pir*), nucleus accumbens core (*AcC*), nucleus accumbens shell (*AcSh*), ventral pallidum (*VP*), olfactory tubercle (*Tu*), islands of Calleja (*ICj*), ventral diagonal band (*VDB*), and lateral septum (*LS*). In the *CPu*, VGLUT2 is somewhat less abundant than VGLUT1. VGLUT2 is present in the globus pallidum (*GP*) (*B*), where VGLUT1 is virtually absent (*F*). In piriform cortex (*Pir*) and islands of Calleja (*ICj*), punctate VGLUT1-ir is stronger and denser than VGLUT2-ir. Note accumulation of low to moderate VGLUT2-ir in the pyramidal cell layer in *C* where VGLUT1-ir is scarce (*G*). Note also some overlap and reciprocity in staining for VGLUT1 and VGLUT2 in the *ICj* (*D*, *H*). Note the absence of VGLUT1-ir and VGLUT2-ir from commissural fiber tracts. *cc*, Corpus callosum; *ac*, anterior commissure. Scale bars: *A*, *C*, 1 mm; *B*, *D*, 500 μ m; *E–H*, 200 μ m.

exceptions to the rule that VGLUT2 was restricted to synapses and absent from perikarya. The nucleus ruber received input of VGLUT2-ir terminals but not of VGLUT1 terminals. Taken together, the wiring of VGLUT2 and VGLUT1 synapses within the extrapyramidal motor pathways displayed marked preferences for specific extrapyramidal subsystems.

Diencephalic pathways

The thalamus exhibited a striking differential complementary staining pattern for VGLUT2 and VGLUT1 (Figs. 5, 7, 8). VGLUT2 synapses were present on many thalamic nuclei, with relative abundance in the paraventricular, reuniens, reticular, paracentral, and anterodorsal thalamic nuclei as well as in the mediocaudal part of the lateral posterior nucleus, and in the posterior intralaminar, peripeduncular, and supragenulate thalamic nuclei. In contrast, VGLUT1 synaptic puncta were more restricted and showed preference to parts of the lateral posterior and ventral posteromedial thalamic nucleus and the dorsal lateral geniculate nucleus, as well as the paratenial nucleus.

In the hypothalamus, VGLUT2 synapses were relatively abundant within most nuclei. In contrast, VGLUT1 was much more restricted to specific diencephalic nuclei, among them the ventral premammillary and ventromedial hypothalamic nucleus. Neuroendocrine centers of the hypothalamus such as the median eminence and the supraoptic and paraventricular nuclei obtained preferential input from VGLUT2 puncta (Fig. 7). Some diencephalic nuclei such as the ventromedial hypothalamic nucleus or the habenular nuclei received dual VGLUT1 and VGLUT2 synaptic input. However, within these nuclei, reciprocity in the microregional distribution and density of VGLUT1- and VGLUT2-ir synaptic puncta was seen (Figs. 7, 8).

The widespread distribution and clear preponderance of VGLUT2 synapses over VGLUT1 synapses in the hypothalamus,

thalamus, and epithalamus conform to previous *in situ* hybridization histochemical data revealing variably abundant VGLUT2 mRNA and little or no VGLUT1 mRNA in most diencephalic nuclei (Hisano et al., 2000). We suggest that diverse diencephalic neuroendocrine, limbic, and sensory functions are regulated by distinct VGLUT1- and VGLUT2-operated extrinsic neurons as well as by VGLUT2-operated intrinsic glutamatergic neurons.

Visual and acoustic systems of the brainstem

Several nuclei of the brainstem visual system receive a strong and preferential input of VGLUT2 synaptic puncta, e.g., the outer border of the dorsolateral geniculate nucleus containing the retinogeniculate input; the superficial gray of the superior colliculus, where retinal ganglionic cells terminate; the lateral posterior thalamic nucleus, which receives afferents from optic areas of the tectum and the occipital cortex; and the medial terminal nucleus of the accessory optic tract, which receives input from the retina (Figs. 5, 9). Here, VGLUT1 synaptic puncta were sparse, if not absent. Thus, the brain stem optic system seems uniquely supplied by VGLUT2 glutamatergic synapses.

Throughout the brain stem auditory system, VGLUT1 clearly dominated. VGLUT1 synaptic puncta were abundant in the cochlear nuclei. Because VGLUT2 synapses are absent from the superficial layer of the dorsal cochlear nucleus, we suggest that the granule cell interneurons are VGLUT1 coded (Fig. 10*E,J*). VGLUT1 puncta are also abundant in the trapezoid body, superior olive, medial geniculate body (Figs. 9, 10), and inferior colliculus (data not shown). The VGLUT2 synaptic input to these nuclei was much lower but still significant. In the ventral cochlear nucleus, however, VGLUT2 puncta were virtually absent (Fig. 10), and thus the bipolar spiral neurons may be preferentially VGLUT1 coded. Thus, the brain stem auditory system is under

Figure 7. Nucleus-specific and differential abundance of VGLUT2 and VGLUT1 in thalamic and hypothalamic nuclei. Note high abundance of VGLUT2-ir (*A*) in the paraventricular thalamic nucleus (*PVA*), reuniens thalamic nucleus (*Re*), reticular thalamic nucleus (*Rt*), paracentral thalamic nucleus (*PC*), and anterodorsal thalamic nucleus (*AD*). Here, VGLUT1-ir (*B*) is almost absent or at low abundance. VGLUT1 (*B*) is moderately abundant in the posterior thalamic nucleus (*PT*) where VGLUT2 (*A*) is sparse. VGLUT1 is virtually absent from the stria medullaris (*sm*) where VGLUT2-ir is sparse. Adjacent frontal sections (*C*, *D*) of the diencephalon demonstrate the abundance of VGLUT2 (*C*) in the anterior hypothalamic nucleus (*AH*) but scarcity in the paraventricular nucleus (*PVN*) and extreme scarcity of VGLUT1-ir in the anterior hypothalamic nucleus (*AH*) and absence from the PVN. Note presence of VGLUT1 (*D*) but absence of VGLUT2 (*C*) in the ventromedial thalamic nucleus (*VM*). Adjacent frontal sections of the hypothalamus (*E*, *F*) demonstrate abundance of VGLUT2 in the lateral hypothalamic nucleus (*LH*), ventromedial hypothalamic nucleus (*VMH*), and dorsomedial hypothalamic nucleus (*DM*) and sparsity of VGLUT1 in the core of the VMH but moderate abundance in its shell. Note that VGLUT2 is less abundant in the shell and more abundant in the core of the VMH. Note some faint staining for VGLUT2 in the median eminence (*ME*). VGLUT1 and VGLUT2 are absent from fiber tracts of the fornix (*f*) and the mammillothalamic tract (*mt*). 3V, Third ventricle. Scale bar, 500 μ m.

dual glutamatergic control by a prominent VGLUT1 synaptic system and a less prominent VGLUT2 synaptic input.

The brainstem vestibular system also received dual VGLUT1 and VGLUT2 synaptic input, with VGLUT1 providing the major input. The multipolar giant-sized cells of the lateral vestibular nucleus were targeted by abundant strongly positive VGLUT1 boutons and less abundant and less frequent VGLUT2 puncta (data not shown).

Brainstem autonomic and motor-sensory pathways

VGLUT2 puncta were widespread throughout the tegmentum and reticular formation, whereas VGLUT1 puncta were less abundant and more limited to specific nuclei (Figs. 9–11). VGLUT2 puncta were enriched in the pain processing area of the dorsal periaqueductal gray (PAG) (Figs. 9C, 9E, 11) and moder-

Figure 8. The distribution of VGLUT2 and VGLUT1 in the epithalamus. High-power micrographs from adjacent sections alternately stained for VGLUT2 (*A*) and VGLUT1 (*B*) demonstrating abundance of VGLUT2 in both the medial habenular nucleus (*MHb*) and the lateral habenular nucleus (*LHb*). Note that VGLUT1-ir in the *MHb* is less dense than VGLUT2-ir. VGLUT1 is virtually absent from *LHb*. Scale bar, 100 μ m.

ately concentrated in the locus coeruleus (Fig. 10A), where VGLUT1 was at very low abundance. Serotonergic raphe nuclei and the various scattered catecholaminergic cell groups in the brainstem were targeted by VGLUT2 rather than by VGLUT1 puncta (data not shown). In the autonomic and pain relay center of the parabrachial nuclei, VGLUT1 and VGLUT2 were sparse and exhibited complementary preference, VGLUT2 in the lateral parabrachial nucleus and VGLUT1 in the medial parabrachial nucleus (Fig. 10A,F). The dorsal motor nucleus of the vagus received substantial input by VGLUT2 puncta but not by VGLUT1 puncta (Fig. 11A–D). VGLUT2 terminals also preferentially supplied the area postrema (data not shown). Synapses in the nucleus of the solitary tract (NTS) were found to be VGLUT2 coded, implying that the presumed glutamatergic primary afferent endings in the NTS preferentially operate with VGLUT2. Among these afferents are the gustatory, baroreceptor, chemoreceptor, and gastrointestinal satiety afferents, suggesting that VGLUT2 rather than VGLUT1 is involved in glutamatergic regulation of cardiorespiratory and cardiovascular homeostasis and in gustatory and gut–brain signaling serving appetite control (Gordon, 1995; Sapru, 1996; Aicher et al., 2000; Sako et al., 2000). The presence of VGLUT2 synapses in the spinal intermediolateral cell column strongly suggests that glutamatergic input to preganglionic autonomic neurons in the spinal cord is specifically VGLUT2 operated. This is in accordance with the expression of VGLUT2 mRNA in oxytocinergic and vasopressinergic neurons of the hypothalamic supraoptic and supraventricular nuclei that are known to project to the preganglionic spinal visceromotor neurons (Hisano et al., 2000).

The pontine nuclei, which are the target of the corticopontine tract and the origin of the pontocerebellar tract, received frequent VGLUT1 but infrequent VGLUT2 puncta (data not shown). The motor trigeminal and facial nuclei were preferentially supplied by VGLUT1 and less by VGLUT2 synaptic puncta (Fig. 10A,F). In contrast, the motor hypoglossal nucleus was receiving VGLUT2 rather than VGLUT1 synaptic input (Fig. 11C,D). VGLUT1 and VGLUT2 synaptic input to sensory relay centers in the brainstem was remarkably differentiated. Presumed glutamatergic nociceptive spinothalamic input with widespread thalamic terminal fields may be VGLUT2 operated, whereas the more restricted thalamic terminations of the dorsal column lemniscal pathway could be specifically VGLUT1 operated. By demonstrating complementary distribution of VGLUT1 and VGLUT2 input to the superficial and deep dorsal horn, as well as to the superficial and deep spinal trigeminal nucleus, we conclude that glutamatergic nociceptive signaling in the substantia gelati-

Figure 9. The distribution of VGLUT2, VGLUT1, and tyrosine hydroxylase (TH) in the mesencephalon and metathalamus. VGLUT2-ir puncta are concentrated in the tectum, with highest levels in the superficial gray layer of the superior colliculus (SUG) and lower levels in the intermediate gray layer of the superior colliculus (InG) and scarcity in the optic nucleus layer of the superior colliculus (Op). VGLUT2-ir puncta are present throughout the tegmentum, including the nucleus ruber (R) and the TH-positive pars compacta of the substantia nigra (SNC) and are enriched in the dorsal periaqueductal gray (PAG) and, in particular, in the medial terminal nucleus of the accessory optic tract (MT) as well as in the mediocaudal part of the lateral posterior nucleus (LPMC), in the posterior intralaminar thalamic nucleus (PIL), in the peripeduncular nucleus (PP), and in the supragenicular thalamic nucleus (SG). Here, VGLUT1-ir is minimal. VGLUT1 staining is present at moderate levels in the ventral medial geniculate nucleus (MGV) where VGLUT2 is minimal. VGLUT1 is minimal throughout the tectum, periaqueductal gray, and tegmentum and virtually absent from the substantia nigra pars compacta (SNC) and pars reticularis (SNR). Weak VGLUT2 staining is present in neuronal perikarya and puncta in the SNR (D) high-power micrograph from area in rectangle in C where VGLUT1 is virtually absent (corresponding area in F). Aq, Mesencephalic aqueduct. Scale bars: A, C, E, 1 mm; B, F, D, 200 μ m.

nosa spinalis and trigeminalis is preferentially related by VGLUT2-operated synapses, whereas mechanoreception at the first synapse of primary afferents in the spinal nucleus proprius, the principal trigeminal nucleus, and the dorsal column nucleus is predominantly VGLUT1 operated (Figs. 10A,F, 11A,B,G,H). This view is supported by our preliminary observation that VGLUT2-ir is prominent in presumed nociceptive small-

diameter spinal and trigeminal afferents, whereas VGLUT1 is relatively abundant in non-nociceptive large-diameter primary sensory neurons (data not shown). Our data suggest differential roles of VGLUT1 and VGLUT2 in spinal and trigeminal nociceptive neurotransmission, with clinical relevance for chronic neuropathic and inflammatory pain.

Cerebellar cortex

The molecular layer of the cerebellar cortex received an exuberant input by VGLUT1 puncta (Fig. 12A,B) and a substantial input by VGLUT2 puncta (Fig. 12C,D) forming strands perpendicular to the surface. The VGLUT1 puncta were so densely packed that individual puncta could only be clearly discerned with high-power confocal microscopy (data not shown). In the granular layer, VGLUT1 and VGLUT2 puncta were confluent and formed rosette-like structures. Also in the granular layer, VGLUT1 was much more abundant than VGLUT2. The VGLUT1 and VGLUT2 input to the somata of the Purkinje cells in the Purkinje layer was comparatively sparse. The extreme abundance of VGLUT1 in the molecular and granular layers of the cerebellar cortex implies that most of the disynaptic input via mossy and parallel fibers is operated by VGLUT1. This is supported by strong expression of BNPI mRNA in the granule cell layer of the cerebellum and in the pontine nuclei that deliver mossy fibers to the granular layer (Ni et al., 1994, 1995). VGLUT2-positive mossy fiber rosettes may arise, in part, from the vestibular nuclei, reticular formation, or spinal cord. The strict perpendicular lining of VGLUT2 synaptic puncta in the molecular layer, typical for synapse lining of climbing fibers, suggests that at least a subset of glutamatergic climbing fibers from the inferior olive, which is known, in part, to convey sensory visual information, is operated by VGLUT2. A VGLUT2 input is obvious at the cell bodies of the Purkinje cells, which are typically targeted by climbing fibers (Fig. 12).

DISCUSSION

Vesicular glutamate transport by VGLUT2

Active vesicular transport of all classical transmitters depends on the H^+ electrochemical gradient ($\Delta\mu_{H^+}$) across the synaptic vesicle membrane that is formed by a vacuolar-type H^+ -ATPase. Proton pumping into vesicles results in a buildup of transmembrane H^+ electric ($\Delta\psi$, positive inside) and chemical H^+ gradients (ΔpH , acidic inside). Initially, the transport of only a few protons generates a charge that limits the ΔpH gradient that can be formed. Dissipation of $\Delta\psi$ is provided by Cl^- ion, which enters vesicles through endomembrane anion channels present in most intracellular organelles, including synaptic vesicles, and provides a charge balance allowing for higher rates of H^+ transport and generation of a ΔpH gradient (al-Awqati, 1995; Szeewczyk, 1998). The relative proportions of ΔpH and $\Delta\psi$ across vesicle membranes therefore may vary greatly depending on the cytoplasmic concentration of the permeating Cl^- ion (Van Dyke, 1988). Altering the Cl^- permeability of vesicles can result in changes in neurotransmitter accumulation (Tamir et al., 1994).

The resting cytoplasmic Cl^- concentration in glutamatergic axon terminals is unknown but varies from 10 mM in the cell body to 35 mM in the processes (Hara et al., 1992; Kuner and Augustine, 2000). Glutamate transport activity of VGLUT2 therefore was examined at four different Cl^- conditions: not present, low (2–4 mM), moderate (16–40 mM), and high (150 mM). The relative contribution of $\Delta\psi$ to the total $\Delta\mu_{H^+}$ is predicted to be maximal when Cl^- is absent or present in low millimolar con-

Figure 10. The distribution of VGLUT2 and VGLUT1 in the pontomedullary brainstem. High abundance of punctate VGLUT2-ir (*B, D*) in the medial superior olive (*MSO*) is observed where VGLUT1-ir is low (*G, I*). VGLUT2-ir is relatively low in the nucleus of the trapezoid body (*TZ*) (*B, C*) where VGLUT1-ir is at high abundance (*G, H*). Note that strongly VGLUT1-positive confluent large puncta encircle immunonegative neuronal cell bodies in the *TZ* (*H*). Strongly positive VGLUT-ir puncta are present in the principal sensory nucleus of the trigeminal nerve (*Pr5*) where VGLUT2-ir is very low. Moderately positive VGLUT1 puncta are present in the motor trigeminal nucleus (*Mo5*) where VGLUT-ir is low. VGLUT-ir and VGLUT2-ir are present at low abundance in the lateral and medial parabrachial nucleus (*LPB, MPB*). Moderate VGLUT2-ir accumulates in the locus coeruleus (*LC*), where VGLUT1-ir is very scarce. VGLUT1-ir and VGLUT2-ir are absent from the pyramidal tract (*pyr*). Adjacent sections (*E, J*) alternately stained for VGLUT2 (*E*) and VGLUT1 (*J*) reveal abundance of VGLUT1-ir in the anterior ventral cochlear nucleus (*VCA*) where VGLUT2 is virtually absent. High-power micrograph (*inset*) from *J* demonstrates strongly positive VGLUT1-ir puncta encircling immunonegative neuronal cell bodies and processes. VGLUT1-ir puncta outnumber VGLUT2-positive puncta in the dorsal cochlear nucleus (*DC*), particularly in the superficial layer where VGLUT2 is absent (*E, J*). Scale bars: *A, E*, 500 μ m; *B, F, I, J*, 200 μ m; *C, D, G, H*, 25 μ m.

centrations, reduced but equivalent to Δ pH under moderate (40 mM) Cl^- conditions, and absent in high Cl^- conditions. In contrast, a Δ pH cannot be measured without Cl^- present, is minimal under low Cl^- concentrations, and maximal under high Cl^- concentrations (Johnson et al., 1981). Glutamate uptake by VGLUT2 is nearly abolished when Cl^- is completely absent from the incubation medium, indicating an essential role for this anion in the uptake mechanism. Maximal levels of glutamate uptake are observed when Cl^- is present at physiological concentrations (4–40 mM) and is not observed under high Cl^- concentrations. Under all Cl^- conditions, uptake is inhibited in the presence of valinomycin that dissipates $\Delta\psi$. One possible role of $\Delta\psi$ may be to drive Cl^- into the synaptic vesicles. The selective activation of VGLUT2-mediated glutamate transport by Cl^- (and Br^-) suggests that entry of Cl^- into the vesicle through an endomembrane anion channel might occur. Glutamate might then be exchanged for intravesicular Cl^- to maintain $\Delta\psi$, which is preserved during glutamate accumulation into brain vesicles (Maycox et al., 1988; Wolosker et al., 1996). Nigericin, which selectively dissipates Δ pH, abolished uptake at 40 mM Cl^- , indicating that Δ pH can also be a major driving force for vesicular glutamate accumulation by VGLUT2.

Relationship between P_i and glutamate transport

Transport of P_i across the plasma membrane by VGLUT1 and VGLUT2 has been demonstrated in the *Xenopus* oocyte system (Ni et al., 1994; Aihara et al., 2000). In this system, the transporters would orient in such a way as to favor endogenous glutamate efflux that could be accompanied by an influx of anions such as P_i or Cl^- ion. Although P_i does not stimulate uptake of glutamate into vesicles by VGLUT2 as does Cl^- , higher P_i concentrations are inhibitory, possibly reflecting an intrinsic conductance to anions similar to the intrinsic permeability of VGLUT1 to high Cl^- concentrations suggested by Bellocchio et al. (2000). It is possible that these proteins might also display a Cl^- conductance in oocytes, as has been shown for NaP_i -1 (Busch et al., 1996; Bröer et al., 1998), and be amenable to biophysical analysis in which possible differences in stoichiometry and relative turnover rates can be assessed (Otis, 2001).

A dual role in mediating P_i transport across the plasma membrane and glutamate uptake into synaptic vesicles would require this protein to be expressed in both places *in vivo*. Although neural activity might increase the steady-state amount expressed at the plasma membrane, most of the VGLUT1 localizes to synaptic vesicles by electron microscopy (Bellocchio et al., 1998).

Figure 11. The distribution of VGLUT2 and VGLUT1 in the lower brainstem. Moderate abundance of fine punctate VGLUT2-ir in superficial spinal trigeminal nucleus (Sp5) marked by arrowheads (*A*, *G*) is observed where VGLUT1-ir is virtually absent (*B*, *H*, arrowheads). VGLUT2 is moderately abundant in the dorsal motor nucleus of the vagus (10), hypoglossal nucleus (12), and reticular formation (Rt), and in the ventral part of the solitary tract (SolV) (*A*, *C*, *E*) where VGLUT1 is virtually absent (*B*, *D*, *F*). VGLUT2-ir is very low in the dorsal solitary tract (SolD). Note preponderance of VGLUT1 in deep Sp5 (*B*, *F*, *H*), in the cuneate (Cu), and in gracile nucleus (GR) where VGLUT2-ir is low. Asterisks mark the central canal. Scale bars: *A*, *B*, 500 μ m; *C*, *D*, 200 μ m; *E*, *F*, 100 μ m; *G*, *H*, 100 μ m.

VGLUT2, like VGLUT1, also colocalizes with synaptic vesicle markers and is restricted to presynaptic terminals by confocal microscopy *in situ*.

Why two isoforms of the vesicular glutamate transporter?

Differences in the molecular roles of Cl^- and H^+ in vesicular glutamate uptake by VGLUT1 and VGLUT2 may exist. VGLUT1 has been suggested to operate solely with $\Delta\psi$ (Bellocchio et al., 2000; Takamori et al., 2000). We show that ΔpH can also be a major driving force for uptake for VGLUT2 under physiological Cl^- conditions. Similar observations reported in isolated synaptic vesicle preparations (Özkan and Ueda, 1998) may be attributed to the unsuspected presence of two isoforms. Differential regulation of VGLUT1 and VGLUT2 gene expression as well as possible differences in rates of intrinsic activity and

Figure 12. The distribution of VGLUT1 and VGLUT2 in the cerebellum. *A*, *B*, Extreme density of intensely stained VGLUT1-positive puncta in the molecular layer (*m*), very sparse VGLUT1 puncta around somata of Purkinje cells in the Purkinje cell layer (*p*), and dense glomerula-like accumulation of strongly stained confluent VGLUT1 puncta in the granular layer (*g*) is observed. *C*, *D*, VGLUT2-ir puncta are much less dense in the molecular layer where they are arranged in a strand-like manner. VGLUT2-ir puncta forming glomerula-like structures in the granular layer (*g*) are less dense than those staining for VGLUT1. Scale bars: *A*, *C*, 500 μ m; *B*, *D*, 100 μ m.

trafficking of these proteins within nerve terminals are open areas of investigation.

The remarkably differentiated pathway- and target-specific dualism of excitatory synaptic circuits suggests unique physiological roles for these proteins. VGLUT1-containing synaptic vesicles are expressed in a subset of synapses of the known glutamatergic pathways that are complementary to VGLUT2-coded innervation of the CNS. Together with mRNA distribution studies (Ni et al., 1994, 1995; Aihara et al., 2000; Hisano et al., 2000), the two isoforms identify classes of excitatory neurons with functions that are involved in learning and memory and in the transfer of peripheral sensory information to the cerebral cortex. VGLUT1 is associated with many synapses that exhibit activity-dependent synaptic plasticity such as long-term potentiation (LTP), whereas VGLUT2 is expressed primarily in sensory and autonomic pathways that display high-fidelity neurotransmission.

The sheer abundance of VGLUT1 over VGLUT2 puncta in certain telencephalic regions of the brain suggests that VGLUT1 is a dominant excitatory system in the corticocortical pyramidal neuron projections and the trisynaptic circuit in the hippocampus. VGLUT1 is uniquely associated with cortical pyramidal neurons involved in higher cognitive function and with cerebellar mossy-parallel fiber input important for skilled motor acquisition. This indicates that LTP during learning is a domain of VGLUT1-governed glutamatergic transmission. VGLUT1 synapses may display a lower release probability in general (Hanse and Gustafsson, 2001; Xu-Friedman et al., 2001), and paired-pulse facilitation and post-tetanic potentiation may include presynaptic mechanisms that increase the probability of glutamate secretion (Salin

et al., 1996; Goussakov et al., 2000; Zakharenko et al., 2001). VGLUT1-encoded transmission may also be intimately involved with information processing at early stages in the auditory system. The dorsal cochlear nucleus (DNC) principal cells, a first-order auditory nucleus, receive various VGLUT1-encoded nonauditory inputs via a cerebellar-like VGLUT1 granule cell circuit located in the superficial layers of the DNC, in addition to their well known auditory inputs. Activity-dependent potentiation has been observed in the granule cell domain and in VGLUT1-encoded pathways of the auditory system and the somatosensory thalamus (Davis et al., 1996; Illing et al., 2000; Weng et al., 2000; Sakai and Suga, 2001); however, a high probability of glutamate release from the VGLUT1-enriched giant synaptic terminals (the calyx of Held) located in the second-order auditory nucleus of the trapezoid body has also been observed (Trussell, 1999; Taschenberger and Gersdorff, 2000). This may be attributable to the presence of multiple release sites (Otis et al., 1996) and multiple calcium channels that are required to release a vesicle at this synapse (Borst and Sakmann, 1998).

VGLUT2 is expressed in many neuronal pathways that characteristically display high-fidelity neurotransmitter release and may be more genetically “hard wired” than VGLUT1-coded pathways. Synaptic transmission between VGLUT2-expressing neurons along the visual and nociceptive sensory pathways to corticocortical association pathways and motor output pathways has a low failure rate and is substantially faster and more reliable than in other cortical laminae (Egger et al., 1999; Feldmeyer et al., 1999; Gil et al., 1999). The predominant glutamatergic thalamic sensory input to the cerebral cortex is to the dendrites of the spiny stellate interneurons in layer IV, which also express VGLUT2 mRNA (Hisano et al., 2000). Potentiation of thalamocortical synapses occurs only during early postnatal development in the rat and involves conversion of silent synapses to functional ones (Crair and Malenka, 1995; Isaac et al., 1997). Potentiation of glutamate release has not been observed in VGLUT2-encoded gustatory afferent endings in the NTS (Bradley and Grabauskas, 1998) or at climbing fiber synapses onto Purkinje neurons that also display high probability release (Dittman et al., 2000; Xu-Friedman et al., 2001). The high activity of VGLUT2-expressing glutamatergic projections of the subthalamic nucleus to the SNC has been implicated as a mechanism driving neurotoxicity in the substantia nigra and the development of Parkinson’s disease (Rodriguez et al., 1998).

It remains to be determined whether a difference in the probability of glutamate neurosecretion is a general function of VGLUT1- versus VGLUT2-encoded synapses. Clearly, calcium fluxes play a central role in regulating release probability (Stevens and Sullivan, 1998; Wu and Borst, 1999). Furthermore, at some synapses, activity-dependent potentiation (and depression) may be entirely a postsynaptic event (Nicoll and Malenka, 1999; Carroll et al., 2001; Kemp and Bashir, 2001). However, VGLUTs play an obvious role in the replenishment of the releasable pool of vesicles and may be subject to differential regulation during intense presynaptic activity.

Authors’ note

While this paper was being reviewed, Fremeau et al. (2001) published a related study that strengthens and complements our hypothesis.

REFERENCES

- Aicher SA, Milner TA, Pickel VM, Reis DJ (2000) Anatomical substrates for baroreflex sympatho-inhibition in the rat. *Brain Res Bull* 51:107–110.
- Aihara Y, Mashima H, Onda H, Hisano S, Kasuya H, Hori T, Yamada S, Tomura H, Yamada Y, Inoue I, Kojima I, Takeda J (2000) Molecular cloning of a novel brain-type Na^+ -dependent inorganic phosphate cotransporter. *J Neurochem* 74:2622–2625.
- al-Awqati Q (1995) Chloride channels of intracellular organelles. *Curr Opin Cell Biol* 7:504–508.
- Bellocchio EE, Hu H, Pohorille A, Chan J, Pickel VM, Edwards RH (1998) The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic neurotransmission. *J Neurosci* 18:8638–8659.
- Bellocchio EE, Reimer RJ, Fremeau Jr RT, Edwards RH (2000) Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science* 289:957–960.
- Borst JG, Sakmann B (1998) Calcium current during a single action potential in a large presynaptic terminal of the rat brainstem. *J Physiol (Lond)* 506:143–157.
- Bradley RM, Grabauskas G (1998) Neural circuits for taste. Excitation, inhibition, and synaptic plasticity in the rostral gustatory zone of the nucleus of the solitary tract. *Ann NY Acad Sci* 855:467–474.
- Bröer S, Schuster A, Wagner CA, Bröer A, Forster I, Biber J, Murer H, Werner A, Lang F, Busch AE (1998) Chloride conductance and P_i transport are separate functions induced by the expression of NaPi-1 in *Xenopus* oocytes. *J Memb Biol* 164:71–77.
- Burger PM, Mehl E, Cameron PL, Maycox PR, Baumert M, Lottspeich F, De Camilli P, Jahn R (1989) Synaptic vesicles immunoprecipitated from rat cerebral cortex contain high levels of glutamate. *Neuron* 7:287–293.
- Busch AE, Schuster A, Waldegger S, Wagner CA, Zempel G, Bröer S, Biber J, Murer H, Lang F (1996) Expression of a renal type I sodium/phosphate transporter (NaPi-1) induces a conductance in *Xenopus* oocytes permeable for organic and inorganic anions. *Proc Natl Acad Sci USA* 93:5347–5351.
- Carlsson A, Waters N, Holm-Waters S, Tedroff J, Nilsson M, Carlsson ML (2001) Interactions between monoamines, glutamate, and GABA in schizophrenia: new evidence. *Annu Rev Pharmacol Toxicol* 41:237–260.
- Carroll RC, Beattie EC, von Zastrow M, Malenka RC (2001) Role of AMPA receptor endocytosis in synaptic plasticity. *Nat Rev Neurosci* 2:315–324.
- Crair MC, Malenka RC (1995) A critical period for long-term potentiation at thalamocortical synapses. *Nature* 375:325–328.
- Davis KA, Miller RL, Young ED (1996) Effects of somatosensory and parallel-fiber stimulation on neurons in dorsal cochlear nucleus. *J Neurophysiol* 76:3012–3024.
- Disbrow JK, Gershten MJ, Ruth JA (1982) Uptake of $\text{L-}[^3\text{H}]$ glutamic acid by crude and purified synaptic vesicles from rat brain. *Biochem Biophys Res Commun* 108:1221–1227.
- Dittman JS, Kreitzer AC, Regehr WG (2000) Interplay between facilitation, depression, and residual calcium at three presynaptic terminals. *J Neurosci* 20:1374–1385.
- Egger V, Feldmeyer D, Sakmann B (1999) Coincidence detection and changes of synaptic efficacy in spiny stellate neurons in rat barrel cortex. *Nat Neurosci* 2:1098–1105.
- Feldmeyer D, Egger V, Lubke J, Sakmann B (1999) Reliable synaptic connections between pairs of excitatory layer 4 neurones within a single “barrel” of developing rat somatosensory cortex. *J Physiol (Lond)* 521:U169–190.
- Floresco SB, Todd CL, Grace AA (2001) Glutamatergic afferents from the hippocampus to the nucleus accumbens regulate activity of ventral tegmental area dopamine neurons. *J Neurosci* 21:4915–4922.
- Fremeau RT, Troyer MD, Pahner I, Nygaard GO, Tran CH, Reimer RJ, Bellocchio EE, Fortin D, Storm-Mathisen J, Edwards RH (2001) The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron* 31:247–260.
- Fuerst TR, Niles EG, Studier FW, Moss B (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc Natl Acad Sci USA* 83:8122–8126.
- Fujiyama F, Furuta T, Kaneko T (2001) Immunocytochemical localization of candidates for vesicular glutamate transporters in the rat cerebral cortex. *J Comp Neurol* 435:379–387.
- Gasnier B (2000) The loading of neurotransmitters into synaptic vesicles. *Biochimie* 82:327–337.
- Gil Z, Connors BW, Amitai Y (1999) Efficacy of thalamocortical and intracortical synaptic connections: quanta, innervation, and reliability. *Neuron* 23:385–397.
- Gordon FJ (1995) Excitatory amino acid receptors in central cardiovascular regulation. *Clin Exp Hypertens* 17:81–90.
- Goussakov IV, Fink K, Elger CE, Beck H (2000) Metaplasticity of mossy fiber synaptic transmission involves altered release probability. *J Neurosci* 20:3434–3441.

- Hanse E, Gustafsson B (2001) Vesicle release probability and preprimed pool at glutamatergic synapses in area CA1 of the rat neonatal hippocampus. *J Physiol (Lond)* 531:481–493.
- Hara M, Inoue M, Yasukura T, Ohnishi S, Mikami Y, Inagaki C (1992) Uneven distribution of intracellular Cl^- in rat hippocampal neurons. *Neurosci Lett* 143:135–138.
- Harteringer J, Jahn R (1993) An anion binding site that regulates the glutamate transporter of synaptic vesicles. *J Biol Chem* 268:23122–23127.
- Hell JW, Maycox PR, Stadler H, Jahn R (1988) Uptake of GABA by rat brain synaptic vesicles isolated by a new procedure. *EMBO J* 7:3023–3029.
- Hisano S, Hoshi K, Ikeda Y, Maruyama D, Kanemoto M, Ichijo H, Kojima I, Takeda J, Nogami H (2000) Regional expression of a gene encoding a neuron-specific Na^+ -dependent inorganic phosphate cotransporter (DNPI) in the rat forebrain. *Mol Brain Res* 83:34–43.
- Huttner WB, Schiebler W, Greengard P, DeCamilli P (1983) Synapsin I (protein I), a nerve terminal-specific phosphoprotein III: its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J Cell Biol* 96:1374–1388.
- Illing RB, Kraus KS, Michler SA (2000) Plasticity of the superior olivary complex. *Microsc Res Tech* 51:364–381.
- Isaac JT, Crari MC, Nicoll RA, Malenka RC (1997) Silent synapses during development of thalamocortical inputs. *Neuron* 18:269–280.
- Jahn R, Südhof TC (1993) Synaptic vesicle traffic: rush hour in the nerve terminal. *J Neurochem* 61:12–21.
- Johnson RG, Carty SE, Scarpa A (1981) Proton: substrate stoichiometries during active transport of biogenic amines in chromaffin ghosts. *J Biol Chem* 256:5773–5780.
- Kemp N, Bashir ZI (2001) Long-term depression: a cascade of induction and expression mechanisms. *Prog Neurobiol* 65:339–365.
- Kuner T, Augustine GJ (2000) A genetically encoded ratio-metric neurotechnique indicator for chloride: capturing chloride transients in cultured hippocampal neurons. *Neuron* 27:447–459.
- Masson J, Sagne C, Hamon M, El Mestikawy S (1999) Neurotransmitter transporters in the central nervous system. *Pharmacol Rev* 51:439–464.
- Maycox PR, Deckwerth T, Hell JW, Jahn R (1988) Glutamate uptake by brain synaptic vesicles. Energy dependence of transport and functional reconstitution in proteoliposomes. *J Biol Chem* 263:15423–15428.
- Moriyama Y, Yamamoto A (1995) Vesicular L-glutamate transporter in microvesicles from bovine pineal glands. *J Biol Chem* 270:22314–22320.
- Naito S, Ueda T (1983) Adenosine triphosphate-dependent uptake of glutamate into protein I-associated synaptic vesicles. *J Biol Chem* 258:696–699.
- Naito S, Ueda T (1985) Characterization of glutamate uptake into synaptic vesicles. *J Neurochem* 44:99–109.
- Ni B, Rostock PR, Nadi NS, Paul SM (1994) Cloning and expression of a cDNA encoding a brain-specific Na^+ -dependent inorganic phosphate cotransporter. *Proc Natl Acad Sci USA* 91:5607–5611.
- Ni B, Wu X, Yan G-M, Wang J, Paul SM (1995) Regional expression and cellular localization of the Na^+ -dependent inorganic phosphate cotransporter of rat brain. *J Neurosci* 15:5789–5799.
- Nicoll RA, Malenka RC (1999) Expression mechanisms underlying NMDA receptor-dependent long-term potentiation. *Ann NY Acad Sci* 868:515–525.
- Nohr D, Schäfer MK-H, Persson S, Romeo H, Nyberg F, Post C, Ekström G, Weihe E (1999) Calcitonin gene-related peptide gene expression in collagen-induced arthritis is differentially regulated in primary afferents and motoneurons: influence of glucocorticoids. *Neuroscience* 93:759–773.
- Otis TS (2001) Vesicular glutamate transporters in cognition. *Neuron* 29:11–14.
- Otis TS, Wu YC, Trussell LO (1996) Delayed clearance of transmitter and the role of glutamate transporters at synapses with multiple release sites. *J Neurosci* 16:1634–1644.
- Özkan ED, Ueda T (1998) Glutamate transport and storage in synaptic vesicles. *Jpn J Pharmacol* 77:1–10.
- Pulvirenti L, Diana M (2001) Drug dependence as a disorder of neural plasticity: focus on dopamine and glutamate. *Rev Neurosci* 12:141–158.
- Rodriguez MC, Obeso JA, Olanow CW (1998) Subthalamic nucleus-mediated excitotoxicity in Parkinson's disease: a target for neuroprotection. *Ann Neurol* 44:S175–188.
- Roseth S, Fykse EM, Fonnum F (1998) Uptake of L-glutamate into synaptic vesicles: competitive inhibition by dyes with biphenyl and amino- and sulphonate acid-substituted naphthyl groups. *Biochem Pharmacol* 56:1243–1249.
- Sakai M, Suga N (2001) Plasticity of the cochleotopic (frequency) map in specialized and nonspecialized auditory cortices. *Proc Natl Acad Sci USA* 98:3507–3512.
- Sakata-Haga H, Kanemoto M, Maruyama D, Hoshi D, Mogi K, Narita M, Okado N, Ikeda Y, Nogami H, Fukui Y, Kojima I, Takeda J, Hisano S (2001) Differential localization and colocalization of two neuron-types of sodium-dependent inorganic phosphate cotransporters in rat forebrain. *Brain Res* 902:143–155.
- Sako N, Harada S, Yamamoto T (2000) Gustatory information of umami substances in three major taste nerves. *Physiol Behav* 71:193–198.
- Salin PA, Malenka RC, Nicoll RA (1996) Cyclic AMP mediates a pre-synaptic form of LTP at cerebellar parallel fiber synapses. *Neuron* 16:797–803.
- Sanes JR, Rubenstein JLR, Nicolas JF (1986) Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J* 5:3133–3142.
- Sapru HN (1996) Carotid chemoreflex neural pathways and transmitters. *Adv Exp Med Biol* 410:357–364.
- Shioi J, Ueda T (1990) Artificially imposed electrical potentials drive L-glutamate uptake into synaptic vesicles of bovine cerebral cortex. *Biochem J* 267:63–68.
- Stevens CF, Sullivan J (1998) Synaptic plasticity. *Curr Biol* 8:R151–R153.
- Storm-Mathisen J, Leknes AK, Bore AT, Vaaland JL, Edminson P, Haug FM, Ottersen OP (1983) First visualization of glutamate and GABA in neurones by immunocytochemistry. *Nature* 301:517–520.
- Stumm R, Culmsee C, Schäfer MK-H, Kriegstein J, Weihe E (2001) Adaptive plasticity in tachykinin and tachykinin receptor expression after focal cerebral ischemia is differentially linked to GABAergic and glutamatergic cerebrocortical circuits and cerebrovascular endothelium. *J Neurosci* 21:798–811.
- Szewczyk A (1998) The intracellular potassium and chloride channels: properties, pharmacology and function. *Mol Membr Biol* 15:49–58.
- Tabb JS, Kish PE, Van Dyke R, Ueda T (1992) Glutamate transport into synaptic vesicles. Roles of membrane potential, pH gradient, and intravesicular pH. *J Biol Chem* 267:15412–15418.
- Takamori S, Rhee JS, Rosenmund C, Jahn R (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* 407:189–194.
- Tamir H, Piscopo I, Liu KP, Hsiung SC, Adlersberg M, Nicolaides M, al-Awqati Q, Nunez EA, Gershon MD (1994) Secretagogue-induced gating of chloride channels in the secretory vesicles of parafollicular cells. *Endocrinology* 135:2045–2057.
- Taschenberger H, Gersdorff H (2000) Fine-tuning an auditory synapse for speed and fidelity: developmental changes in presynaptic waveform, EPSC kinetics, and synaptic plasticity. *J Neurosci* 20:9162–9173.
- Trussell LO (1999) Synaptic mechanisms for coding timing in auditory neurons. *Annu Rev Physiol* 61:477–496.
- Van Dyke RW (1988) Proton pump-generated electrochemical gradients in rat liver multivesicular bodies. Quantitation and effects of chloride. *J Biol Chem* 263:2603–2611.
- Varoqui H, Erickson JD (1997) Vesicular neurotransmitter transporters: potential sites for the regulation of synaptic function. *Mol Neurobiol* 15:165–192.
- Weihe E, Eiden LE (2000) Chemical neuroanatomy of the vesicular amine transporters. *FASEB J* 14:2435–2449.
- Weng HR, Lee JI, Lenz FA, Schwartz A, Vierck C, Rowland L, Dougherty PM (2000) Functional plasticity in primate somatosensory thalamus following chronic lesion of the ventral lateral spinal cord. *Neuroscience* 101:393–401.
- Werner A, Moore ML, Mantei N, Biber J, Semenza G, Murer H (1991) Cloning and expression of cDNA for a Na/Pi cotransport system of kidney cortex. *Proc Natl Acad Sci USA* 88:9608–9612.
- Wolosker H, de Souza DO, de Meis L (1996) Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. *J Biol Chem* 271:11726–11731.
- Wu LG, Borst JG (1999) The reduced release probability of releasable vesicles during recovery from short-term synaptic depression. *Neuron* 23:821–832.
- Xie XS, Crider BP, Stone DK (1989) Isolation and reconstitution of the chloride transporter of clathrin-coated vesicles. *J Biol Chem* 264:18870–18873.
- Xu-Friedman MA, Harris KM, Regehr WG (2001) Three-dimensional comparison of ultrastructural characteristics at depressing and facilitating synapses onto cerebellar Purkinje cells. *J Neurosci* 21:6666–6672.
- Zakharenko SS, Zablow L, Siegelbaum SA (2001) Visualization of changes in presynaptic function during long-term synaptic plasticity. *Nat Neurosci* 4:711–717.