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Synaptic and vesicular co-localization of the glutamate transporters VGLUT1 and VGLUT2 in the mouse hippocampus

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Abstract

Vesicular glutamate transporters (VGLUTs) are essential to glutamatergic synapses and determine the glutamatergic phenotype of neurones. The three known VGLUT isoforms display nearly identical uptake characteristics, but the associated expression domains in the adult rodent brain are largely segregated. Indeed, indirect evidence obtained in young VGLUT1-deficient mice indicated that in cells that co-express VGLUT1 and VGLUT2, the transporters may be targeted to different synaptic vesicles, which may populate different types of synapses formed by the same neurone. Direct evidence for a systematic segregation of VGLUT1 and VGLUT2 to distinct synapses and vesicles is lacking, and the mechanisms that may convey this segregation are not known. We show here that VGLUT1 and VGLUT2 are co-localized in many layers of the young hippocampus. Strikingly, VGLUT2 co-localizes with VGLUT1 in the mossy fibers at early stages. Furthermore, we show that a fraction of VGLUT1 and VGLUT2 is carried by the same vesicles at these stages. Hence, hippocampal neurones co-expressing VGLUT1 and VGLUT2 do not appear to sort them to separate vesicle pools. As the number of transporter molecules per vesicle affects quantal size, the developmental window where VGLUT1 and VGLUT2 are co-expressed may allow for greater plasticity in the control of quantal release.

Keywords: excitatory terminals, postnatal development, quantal size, synaptic vesicles, vesicular glutamate transporter.

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Glutamate, the major excitatory neurotransmitter in the brain, is loaded into synaptic vesicles by proton-dependent transporters and is stored there until Ca^{2+}-dependent exocytosis triggers its release (Gasnier 2000). So far, three subtypes of vesicular glutamate transporters (VGLUT1–3) have been identified and studied. VGLUT1, 2 and 3 show a high degree of structural identity and, as predicted by earlier studies (Ozkan and Ueda 1998), their glutamate transport properties appear to be virtually identical (for a review see Fremeau *et al*. 2004b). Ectopic expression of either VGLUT1 or VGLUT2 in GABAergic cells is sufficient for the induction of glutamate release (Takamori *et al*. 2000a, 2001). Although a similar observation has not been reported in the case of VGLUT3, the secretion of glutamate by VGLUT3-expressing inhibitory neurones in a slice preparation was recently demonstrated (Gillespie *et al*. 2005). Therefore, VGLUTs are specific molecular markers of vesicular glutamate release.

In the adult central nervous system, VGLUT1 is mainly expressed by excitatory neurones within the cerebral and cerebellar cortices, as well as in the hippocampus and thalamus. In contrast, VGLUT2 is expressed mostly by subcortical glutamatergic neurones from the thalamus to the spinal cord (Herzog *et al*. 2001; Kaneko and Fujiyama 2002; Varoqui *et al*. 2002; Fremeau *et al*. 2004b). Thus, VGLUT1 and VGLUT2 show almost mutually exclusive expression patterns and together cover the complete population of conventional glutamatergic neurones. Relatively few occurrences of co-localization of the two proteins have been

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Abbreviations used: CA1, CA2 and CA3, Ammon's horn region 1, 2 and 3; GLUR2/3, AMPA-type glutamate receptor subunits 2 and 3; P, postnatal day; PBS, phosphate-buffered saline; PSD95, postsynaptic density protein of 95 kDa; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Syp1, Synaptophysin 1; VGLUT, vesicular glutamate transporter.

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reported for the adult brain (Sakata-Haga *et al*. 2001; Hisano *et al*. 2002), and co-localization to the same synaptic vesicles has not been described to date (Takamori *et al*. 2001). More recently, developmental studies and the analysis of VGLUT1 knock-out mice showed that VGLUT2 is expressed in VGLUT1 expression domains during early postnatal development (Miyazaki *et al*. 2003; Boulland *et al*. 2004; Fremeau *et al*. 2004a; Wojcik *et al*. 2004; Danik *et al*. 2005; Gras *et al*. 2005).

The fact that during development the regional co-expression of VGLUT1 and VGLUT2 can be detected throughout the brain raises the possibility that co-localization of the two transporters in the same synapses plays a role during synaptic network development. Indeed, VGLUT1 and VGLUT2 co-localize to the same synapses in primary neuronal cultures (Wojcik *et al*. 2004; De Gois *et al*. 2005), and changes in the expression levels of VGLUTs lead to changes in the size of the quanta of glutamate released at synapses (Daniels *et al*. 2004; Wojcik *et al*. 2004; Wilson *et al*. 2005), and VGLUT1 and VGLUT2 genes are regulated independently upon alteration of the network activity of cortical neuronal cultures (De Gois *et al*. 2005). However, studies on the phenotypic characteristics of VGLUT1-deficient neurones *in situ* indicated that VGLUT1 and VGLUT2 are targeted to separate synapses with distinct properties (Fremeau *et al*. 2004a). In the present study we resolve the discrepancy between published studies on the subcellular VGLUT1 and VGLUT2 expression patterns, by showing directly that VGLUT1 and VGLUT2 are not only targeted to the same synaptic terminals in certain neuronal populations of the developing hippocampus, but are also targeted to the same synaptic vesicles.

Materials and methods

Animals

Young and adult VGLUT1 knock-out and wild-type littermate mice (Wojcik *et al*. 2004) were housed in an animal room with a 12-h light/dark cycle with food and water provided *ad libitum*. After weaning, VGLUT1 knock-out mice were fed with moist food and water placed at the bottom of the cage. All efforts were undertaken to minimize the number of animals used and their suffering. All procedures involving animals and their care were conducted in accordance with the institutional guidelines that are in compliance with national and international laws and policies (European Community Council directive #86/609, October 19, 1987).

Cell culture

Neuroendocrine BON cell lines stably expressing either the rat VGLUT1 (clone AL18) or the rat VGLUT2 (clone AP10) (Herzog *et al*. 2001) were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/nutrient mix F-12 (1 : 1) (Invitrogen, Carlsbad, CA, USA), supplemented with 7.5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 600 μg/mL G418.

Immunofluorescence histochemistry

Animals were deeply anaesthetized with tribromoethanol. After a brief rinse with normal saline, each mouse was perfused transcardially with cold 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 10 min. The brains were quickly removed, placed overnight in 20% sucrose/0.02 M potassium PBS for cryoprotection, and subsequently frozen in isopentane at −30°C. Alternatively, animals were perfused with 0.9% NaCl containing $NaNO_2$ (1 g/L) and then brains were dissected and rapidly frozen in isopentane at −30°C. Either sagittal or coronal 14-μm frozen cryostat sections were collected and stored at −80°C. Sections from saline-perfused animals were post-fixed in methanol at −20°C and washed three times with PBS. Sections were blocked in PBS containing 2% gelatin and 0.25% Triton X-100 for 30 min at room temperature (22°C). Thereafter, sections were incubated at 4°C overnight with: polyclonal antibodies to VGLUT2 (1 : 2000–1 : 4000) from either rabbit (Synaptic Systems, Göttingen, Germany; DN2L3Bf from Herzog *et al*. 2001) or guinea pig (Chemicon, Temecula, CA, USA); polyclonal antibodies to VGLUT1 (1 : 2000) from rabbit (Synaptic Systems); polyclonal antibodies to GLUR2/3 (1 : 1000) from rabbit (Chemicon); or monoclonal mouse antibodies to either postsynaptic density protein of 95 kDa (PSD95) (1 : 1000; Upstate Biotechnology, Lake Placid, NY, USA) or Synaptophysin 1 (Syp1, 1 : 1000; Sigma, Saint Louis, MO, USA). The antibodies were diluted in the blocking buffer. After three rinses in PBS, the sections were incubated for 2 h at room temperature with Alexa488-, Alexa555- or Alexa633-labelled goat anti-rabbit, anti-guinea pig or anti-mouse Immunoglobulin G (IgG) secondary antibodies (Molecular Probes, Eugene, OR, USA). The sections were then rinsed three times in PBS and mounted with Mowiol. Overview images of hippocampi were captured with a BX61 conventional microscope (Olympus, Hamburg, Germany) equipped with a FluoViewII Cooled CCD camera and the AnalySIS software package (SIS, Münster, Germany). High-magnification grayscale pictures were collected with either a TCS-SP2 laser-scanning microscope (Leica, Wetzlar, Germany) or an LSM510 laser-scanning microscope (Zeiss, Oberkochen, Germany). Narrow windows of emission wavelength were set for acquisition in order to avoid interference between fluorescence channels. Images are displayed as false-colour RGB.

Immunoisolation of synaptic vesicles and western blotting

For the immunoisolation of synaptic vesicle fractions, brains were removed on ice and all subsequent steps carried out at 4°C. For each experiment, the brains from four either wild-type or VGLUT1$^{-/-}$ postnatal day 10 (P10) mice were homogenized in 8 mL of buffer containing 300 mM sucrose, 5 mM HEPES-KOH (pH 7.4), 5 mM NaK-tartrate, 2 mM $MgCl_2$, 16 μM $NaVO_4$, 2 μg/mL pepstatin A and 0.2 mM phenylmethylsulphonyl fluoride. Similarly, 150 cm^2 of confluent BON cells were scraped in 10 mL of homogenization buffer. Cell membranes were cracked by drawing 5 mL of homogenate through a 27-G (diameter 0.4 mm) needle back and forth 10 times. Homogenates from either brain or cracked cells were centrifuged at 35 000 *g* for 20 min, and equal quantities of the resulting supernatant were incubated with 5 μL of antibody conjugated Eupergit C1Z methacrylate microbeads [rabbit anti-VGLUT1, rabbit anti-VGLUT2, mouse anti-Syp1 (C7.2) and glycine-coated control beads; Takamori *et al*. 2001]. For the control experiment with BON cell supernatants, VGLUT1- and VGLUT2-

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positive samples were incubated either separately or mixed together in proportions mimicking the relative concentration of each isoform in the P10 brain tissue, as judged by western blotting. The total protein concentration was adjusted with bovine serum albumin (BSA). The beads were incubated with the supernatant for 20–30 min, pelleted by centrifugation in an Eppendorf centrifuge (1 min at 10 000 *g*), washed four times with homogenization buffer, and finally resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis [sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE)] sample buffer. Samples were heated to 95°C for 3 min and standard SDS–PAGE and immunoblotting were performed with equal quantities of each sample. Antibodies used to analyse the bead-bound material were polyclonal guinea pig anti-VGLUT1 (AB5905; Chemicon), polyclonal guinea pig anti-VGLUT2 (AB5907; Chemicon), monoclonal mouse anti-Syp1 (C7.2; Synaptic Systems), and monoclonal mouse anti-Rab3 (C41.1; Synaptic Systems). Immunoreactive bands were visualized with enhanced chemoluminescence (Amersham Biosciences, Little Chalfont, UK).

Results

Expression of VGLUT2 in hippocampal mossy fibers

VGLUT2 immunostaining was performed on P10 (not shown), P17 and P60 sagittal brain sections of both wild-type and VGLUT1$^{-/-}$ mice (Fig. 1). At all ages examined, the distribution of VGLUT2 was found to be identical in wild-type and VGLUT1$^{-/-}$ mice. Therefore, VGLUT1$^{-/-}$ sections are not shown. As expected, at all stages, VGLUT2 antibodies strongly stained (i) large terminals in the outer granule layer of the dentate gyrus and in CA2, and (ii) small varicosities in the molecular layer of the dentate gyrus and in the *stratum lacunosum moleculare* of the Ammon's horn region 1 (CA1 region; see Figs 1a and b; Halasy *et al*. 2004). Surprisingly, in young mice (P3, P10, not shown, and P17) antibodies to VGLUT2 also strongly stained the mossy fibre pathway that arises from granule cells of the dentate gyrus to contact CA3 pyramidal cell dendrites in the *stratum lucidum* (Figs 1a and c, arrowheads). At P17, the VGLUT2 staining co-localizes with VGLUT1 and Syp1 in large terminals of the *stratum lucidum* (Fig. 1c, arrowheads). Small VGLUT2 varicosities that do not stain for VGLUT1 were also detected. These varicosities show either low or no staining for Syp1 (Fig. 1c, arrows).

Immunodetection of VGLUT2 at VGLUT1-positive terminals in CA1

At P10, P17 and P60 the staining for VGLUT2 seems extremely low in the *stratum radiatum* and *stratum oriens*

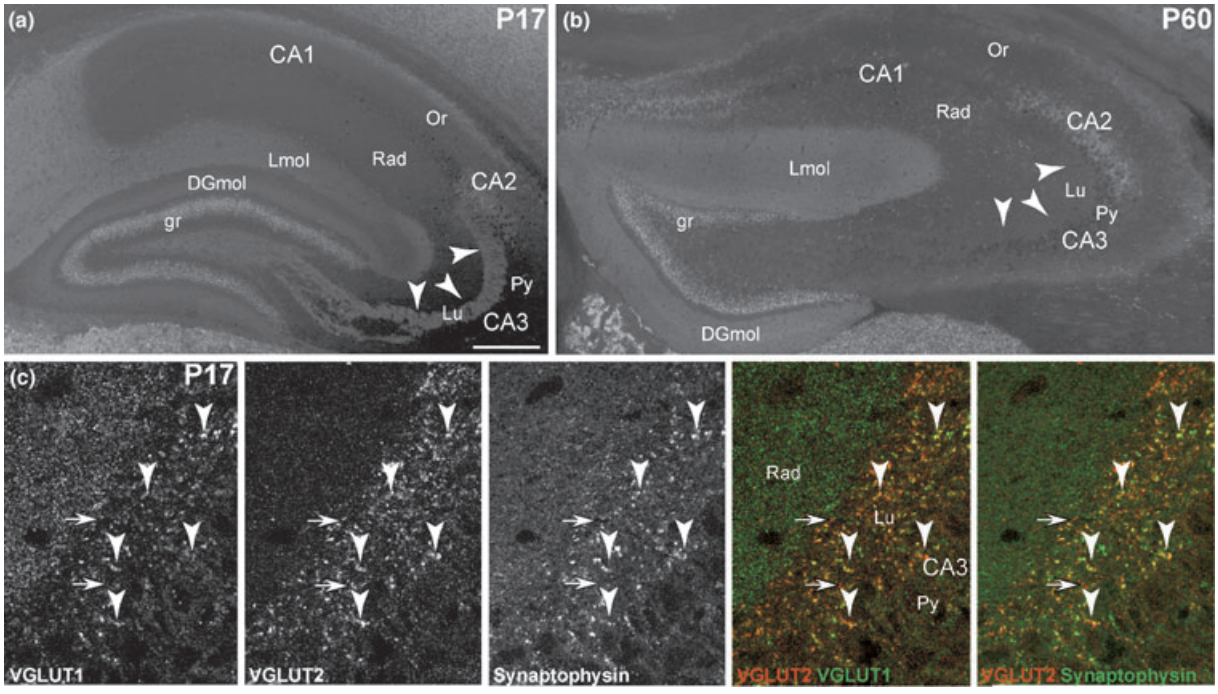

Fig. 1 Vesicular glutamate transporter 2 (VGLUT2) expression in the young and adult hippocampus. (a) Immunofluorescence detection of VGLUT2 in the wild-type mouse hippocampus at postnatal day 17 (P17). Arrowheads point to the mossy fibre pathway. (b) Immunofluorescence detection of VGLUT2 in the wild-type adult mouse hippocampus. Arrowheads point to the mossy fibre pathway. (c) Co-localization of VGLUT1, VGLUT2 and Synaptophysin 1 (Syp1) in the *stratum lucidum* of P17 mouse hippocampus (false colour). Arrowheads point to VGLUT1- and VGLUT2-positive large puncta. Arrows point to VGLUT2-positive small puncta. CA1, CA2 and CA3, Ammon's horn region 1, 2 and 3; Dgmol, dentate gyrus *stratum moleculare*; gr, granule cell layer; Lmol, *stratum lacunosum moleculare*; Lu, *stratum lucidum*; Or, *stratum oriens*; Py, *stratum pyramidale*; Rad, *stratum radiatum*. Scale bars: 200 μm (a and b) and 25 μm (c).

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layers of the CA1 region of the hippocampus (Figs 1a and b). However, at high magnification we observed thin and small varicosities (for *stratum radiatum* see Fig. 2, middle panel). These varicosities were detected with three independent anti-VGLUT2 sera in both wild-type and VGLUT1$^{-/-}$ mice (not shown), but not when sections were only exposed to secondary antibodies (not shown). Finally, these puncta were detected in both paraformaldehyde- and methanol-fixed tissues, however, the signal to noise ratio was higher when methanol fixation was performed.

Most of these VGLUT2 puncta co-localize with the presynaptic marker Syp1 (Fig. 2a, arrowheads). However, a few puncta do not seem to be localized presynaptically, as they are segregated from Syp1 (Fig. 2a, arrows). Postsynaptic markers such as the PSD95 and the glutamate receptor subunits GLUR2 and 3 did not co-localize with VGLUT2 varicosities, but indeed frequently apposed VGLUT2 varicosities (Figs 2b and c, double arrowheads). Finally, at P10, P17 and P60 most of the VGLUT2 puncta also stained positively for VGLUT1, which is known to be the main VGLUT isoform expressed in hippocampal glutamatergic cells and synapses (Figs 2d and e, arrowheads; P60 not shown). However, as expected, most VGLUT1-positive synapses did not contain VGLUT2 (Figs 2d and e).

Immunoisolation of synaptic vesicles

As VGLUT1 and VGLUT2, when present in the same synapse, could be sorted to either the same or separate vesicle pools, we immunoisolated either VGLUT1- or VGLUT2-positive vesicle populations from young mice

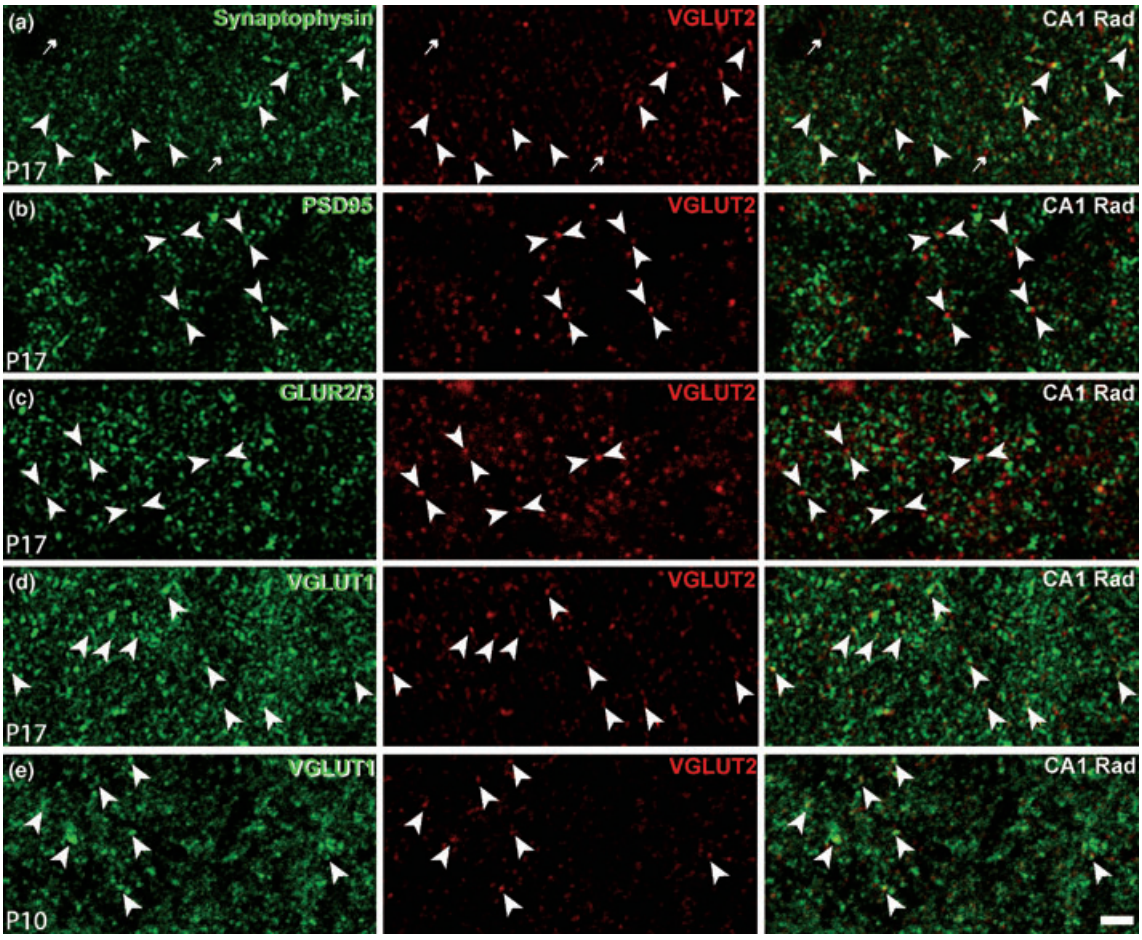

Fig. 2 Localization of vesicular glutamate transporter 2 (VGLUT2) at Ammon's horn region 1 (CA1) synapses and co-localization with VGLUT1. (a) VGLUT2 and Synaptophysin 1 (Syp1) double immunofluorescence staining in CA1 stratum radiatum. Arrowheads point to co-localizing puncta. Some VGLUT2 puncta are not positive for Syp1 (see arrows). (b) VGLUT2 and postsynaptic density protein of 95 kDa (PSD95) double-immunofluorescence staining in CA1 *stratum radiatum*. Double arrowheads point to apposing puncta. (c) VGLUT2 and AMPA-type glutamate receptor subunits 2 and 3 (GLUR2/3) double-immunofluorescence staining in CA1 *stratum radiatum*. Double arrowheads point to apposing puncta. (d) VGLUT2 and VGLUT1 double-immunofluorescence staining in CA1 *stratum radiatum* at P17. (e) VGLUT2 and VGLUT1 double-immunofluorescence staining in CA1 *stratum radiatum* at postnatal day 10 (P10). Arrowheads point to co-localizing puncta. CA1, Ammon's horn region 1; Rad, *stratum radiatum*. Scale bar: 2 μm.

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and tested for co-purification of the two transporters. Immunoisolation of synaptic vesicles was performed with beads coated with anti-VGLUT1, anti-VGLUT2 or anti-Syp1 antibodies. Glycine-coated beads were used as control. The immunobeads were incubated with crude P10 whole brain synaptic vesicle preparations in the absence of detergent. Material that remained bound to the immunobeads after several washing steps was analysed by standard western blotting (Fig. 3). The immunobeads probably isolate protein in various membraneous compartments, including endosomes and synaptic vesicles, but, at least in the case of anti-Viaat beads, it has been demonstrated by electron microscopic analysis that a large part of the material isolated by this method consists of bead-bound synaptic vesicles (Takamori *et al*. 2000b).

The glycine-coated control beads frequently resulted in background artifacts. In particular, Rab3 appeared to stick to the beads themselves (see control beads in Fig. 3a). However, both anti-VGLUT1 and anti-VGLUT2 beads immunoisolated not only the targeted but also the respective other isoform in quantities that were above background levels, indicating that VGLUT1 and VGLUT2 are present on the same vesicles (Fig. 3a). Compared with the quantity of VGLUT1 and VGLUT2 isolated with anti-Syp1 beads, which should bind both VGLUT1 and VGLUT2 vesicles, less of each transporter isoform was isolated by the anti-VGLUT beads which did not target that isoform directly. Vesicle preparations from VGLUT1 knock-out animals were used as controls, and here the anti-VGLUT1 beads were devoid of VGLUT1, VGLUT2 and Syp1, whereas anti-VGLUT2 beads and anti-Syp1 beads each immunoisolated both VGLUT2 and Syp1 (Fig. 3b). If detergent was included in the vesicle preparation to disrupt lipid membranes, only the protein recognized by the respective bead-coupled antibody was detected in the bead fractions (Fig. 3c). To confirm that the co-isolation of VGLUT1 and VGLUT2 from brain extract is the result of the presence of both isoforms on the same vesicles, we also isolated VGLUT1 and VGLUT2

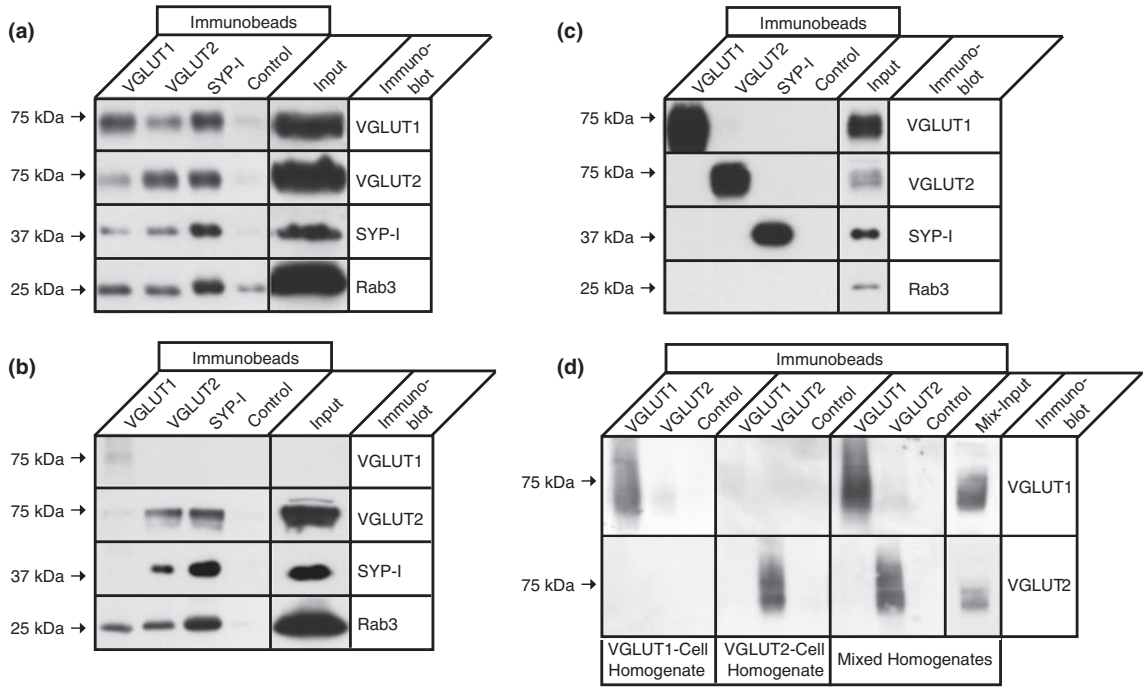

Fig. 3 Immunoisolation of synaptic vesicles containing both vesicular glutamate transporter 1 and 2 (VGLUT1 and VGLUT2) from P10 whole brain. (a) Synaptic vesicle fractions from wild-type mice. Antibodies conjugated to beads are listed above the lanes; antibodies used for western blotting are listed on the right. Note that less VGLUT1 is immunoisolated with anti-VGLUT2 beads than with anti-VGLUT1 beads, and the opposite is true for VGLUT2. (b) Synaptic vesicle fractions from VGLUT1$^{-/-}$ mice. As expected, anti-VGLUT1 beads did not isolate VGLUT1, VGLUT2 or Synaptophysin 1 (Syp1) from VGLUT1$^{-/-}$ samples. Note that anti-VGLUT1 bead samples from VGLUT1$^{-/-}$ mice probed with the guinea pig anti-VGLUT1 antibody consistently gave a background smear covering the entire lane, part of which can be seen in the panel. An apparent band is part of the smear but migrates closer to the 75-kDa molecular marker than VGLUT1 sample. For unknown reasons Rab3 was frequently detected on botha control beads and anti-VGLUT1 beads in VGLUT1$^{-/-}$ samples. (c) In the presence of detergent, immunobeads only bound the protein targeted by the conjugated antibody. (d) Immunoisolation of VGLUT1 and VGLUT2 from homogenates prepared from two BON cell lines that stably express VGLUT1 and VGLUT2, respectively. No co-isolation of VGLUT1 and VGLUT2 was seen when the immunobeads were incubated with a mixture of both homogenates. Positions of the molecular weight marker are indicated on the left.

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from homogenates prepared from two BON cell lines stably expressing either VGLUT1 or VGLUT2, as well as a mixture of both homogenates (Fig. 3d). In this control experiment VGLUT1 and VGLUT2 are not present in the same membranous compartment, and consequently co-isolation should not occur. Indeed, VGLUT1 and VGLUT2 were not co-isolated from a mixture of both BON cell-line homogenates (Fig. 3d). Taken together, our results indicate that a subpopulation of vesicles in P10 mouse brain contains both VGLUT1 and VGLUT2.

Discussion

The question as to whether neurones that co-express VGLUT1 and VGLUT2 sort these transporters either to separate synapses or the same synapses but separate vesicle pools is of key importance in understanding why neurones express both VGLUT genes and go through a developmental transition from VGLUT2 to VGLUT1 expression. In the present study we show that VGLUT2 is transiently expressed in hippocampal mossy fibers between P3 and early adult stages. During this developmental period, VGLUT2 and VGLUT1 are co-localized in mossy fibre terminals (Fig. 1). In the CA1 *stratum radiatum* and *stratum oriens*, a subpopulation of VGLUT1-positive terminals weakly stains for VGLUT2 throughout postnatal life (Fig. 2). Finally, using an immunoisolation technique, we show that at early postnatal stages a subpopulation of synaptic vesicles contains both vesicular glutamate transporters (Fig. 3).

Co-localization of VGLUT1 and VGLUT2 at synapses of the young and adult hippocampus

In the adult mammalian brain, the trisynaptic hippocampal networks have been thoroughly characterized to be a typical VGLUT1 expression domain (Bellocchio *et al*. 1998; Fremeau *et al*. 2001; Herzog *et al*. 2001; Kaneko and Fujiyama 2002; Varoqui *et al*. 2002). Consistent with our finding of VGLUT2 expression in developing mossy fibers are the recent reports of a strong expression of VGLUT2 in the granule cells of the dentate gyrus during the early stages of postnatal life, which decreases to become almost undetectable at adult stages (Miyazaki *et al*. 2003; Boulland *et al*. 2004; Fremeau *et al*. 2004a; Danik *et al*. 2005; Gras *et al*. 2005). Our observations confirm and complement the results from Nakamura and collaborators published during the preparation of our manuscript (Nakamura *et al*. 2005). Interestingly, VGLUT2 is targeted to two kinds of terminals in the *stratum lucidum* that most likely correspond to large VGLUT1-positive mossy fibre terminals contacting CA3 pyramidal cells, and to small filopodial VGLUT1-negative terminals that contact CA3 GABAergic interneurones (Chicurel and Harris 1992; Acsady *et al*. 1998). Overall, our data correlate well with observations of differential regulation of synaptic function and plasticity in the mossy fibre pathway throughout development and according to the respective target of the granule cells (Battistin and Cherubini 1994; Domenici *et al*. 1998; Bischofberger and Jonas 2002). Further investigations will be necessary to fully elucidate the significance of VGLUT1 and VGLUT2 expression in developing mossy fibers.

VGLUT1/VGLUT2-positive synapses observed in the CA1 region stem from pyramidal neurones of the CA3 layer of the hippocampus. Like mossy fibers, CA3 pyramidal cells were reported to express VGLUT2 in addition to VGLUT1 at early stages of development (Miyazaki *et al*. 2003; Boulland *et al*. 2004; Fremeau *et al*. 2004a; Danik *et al*. 2005; Gras *et al*. 2005). However, in CA3 cells a low but significant level of VGLUT2 mRNA is still present at adult stages (Danik *et al*. 2005). These observations are consistent with our finding that a subpopulation of Schaffer collateral terminals remains positive for both VGLUT1 and VGLUT2 at all stages of development. These terminals may correspond to the subpopulation of small VGLUT1/VGLUT2-positive terminals in the *stratum radiatum* of CA1 that were detected by post-embedding electron miscroscopic analysis (Boulland *et al*. 2004). In contrast to our findings, previous studies did not report any co-localization of VGLUT1 and VGLUT2 in the CA1 area (Fremeau *et al*. 2004a; Nakamura *et al*. 2005). This discrepancy might be the result of the very low level of expression of VGLUT2 in the CA1 area, which, although detectable in paraformaldehyde-fixed tissue, is better visualized with methanol fixation. Interestingly, we also identified some non-synaptic VGLUT2-positive puncta in the CA1 region. These are most likely attributable to an involvement of VGLUT2 in retrograde (Harkany *et al*. 2004) and/or astrocytic (Bezzi *et al*. 2004; Montana *et al*. 2004; Zhang *et al*. 2004) glutamate signalling pathways.

Co-localization at the vesicle level

In the present study, we report synaptic co-localization of VGLUT1 and VGLUT2 in the CA3 and CA1 region of the hippocampus. Synaptic co-localization at early postnatal stages has also been reported for the CA1 *stratum lacunosum moleculare*, dentate gyrus *stratum moleculare*, cortex, thalamus (unpublished observation, and Nakamura *et al*. 2005), cerebellar mossy and parallel fibers (Hisano *et al*. 2002; Miyazaki *et al*. 2003), and in primary cortical and hippocampal neurone cultures (Wojcik *et al*. 2004; De Gois *et al*. 2005). Hence, many neurones that co-express VGLUT1 and VGLUT2 during development do not discriminate between the two transporters by sorting them to separate synapses. Our finding that a subpopulation of synaptic vesicles immunoisolated from P10 mouse brain contains both VGLUT1 and VGLUT2 implies that within one synapse both transporters are also sorted to the same vesicles. This notion is supported by our analyses of cultured VGLUT1-deficient hippocampal neurones, some of which show detectable but strongly reduced vesicular glutamate release

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(Wojcik *et al*. 2004; see below). During postnatal development most of the VGLUT2 mRNA and protein disappear progressively from VGLUT1 expression domains (Miyazaki *et al*. 2003; Fremeau *et al*. 2004a; Danik *et al*. 2005; Gras *et al*. 2005; Nakamura *et al*. 2005). Therefore if VGLUT1- and VGLUT2-positive vesicles exist in the adult brain, they may be too rare to be detected reliably above background levels with current biochemical techniques (Takamori *et al*. 2001).

Functional implications

We had previously detected a low level of VGLUT2 protein in synapses of hippocampal neurones cultured from VGLUT1 knock-out mice. This low level of VGLUT2 expression correlated with reduced quantal size in VGLUT1 knock-out neurones, a finding that can only be explained if VGLUT1 and VGLUT2 are sorted to the same vesicles in wild-type neurones (Schuske and Jorgensen 2004; Wojcik *et al*. 2004). One caveat with our previous analyses was that the data were obtained from cultured neurones, which may not fully represent the conditions found *in vivo*. Indeed, another study analysing VGLUT1 knock-out neurones using slice electrophysiology did not reveal a reduction in quantal size, a finding that led to the conclusion that VGLUT1 and VGLUT2 are not only sorted to separate release sites, but also to different vesicles (Fremeau *et al*. 2004a). Furthermore, it was demonstrated recently that in *Drosophila melanogaster* a single functional transporter unit is sufficient for vesicle filling (Daniels *et al*. 2006). However, in contrast to flies, which posses only a single VGLUT gene, mammals have three distinct VGLUT isoforms. This discrepancy may indicate that the mammalian nervous system developed a more finely tuned regulation of vesicular glutamate transport. Our study shows that VGLUT1- and VGLUT2-positive synapses are a prominent feature of the developing hippocampus, and that synaptic vesicles carrying both VGLUT1 and VGLUT2 can be isolated from developing mouse brain.

Modulation of VGLUT levels was shown to affect quantal size in cultured neurones as well as at the *D. melanogaster* neuromuscular junction (Daniels *et al*. 2004; Wilson *et al*. 2005), and VGLUT1 and VGLUT2 genes were recently found to undergo opposite regulation when network activity in neurone cultures was suppressed pharmacologically (De Gois *et al*. 2005). Hence, the developmental period of VGLUT1/VGLUT2 co-expression may be a time window of high presynaptic quantal size plasticity, and neurones expressing VGLUT1, VGLUT2 or both may show distinct responses to regulatory inputs during network development.

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