Lack of Evidence for Vesicular Glutamate Transporter Expression in Mouse Astrocytes

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The concept of a tripartite synapse including a presynaptic terminal, a postsynaptic spine, and an astrocytic process that responds to neuronal activity by fast gliotransmitter release, confers to the electrically silent astrocytes an active role in information processing. However, the mechanisms of gliotransmitter release are still highly controversial. The reported expression of all three vesicular glutamate transporters (VGLUT1–3) by astrocytes suggests that astrocytes, like neurons, may release glutamate by exocytosis. However, the demonstration of astrocytic VGLUT expression is largely based on immunostaining, and the possibility of nonspecific labeling needs to be systematically addressed. We therefore examined the expression of VGLUT1–3 in astrocytes, both in culture and *in situ*. We used Western blots and single-vesicle imaging by total internal reflection fluorescence microscopy in live cultured astrocytes, and confocal microscopy, at the cellular level in cortical, hippocampal, and cerebellar brain slices, combined with quantitative image analysis. Control experiments were systematically performed in cultured astrocytes using wild-type, VGLUT1–3 knock-out, VGLUT1^{Venus} knock-in, and VGLUT2-EGFP transgenic mice. In fixed brain slices, we quantified the degree of overlap between VGLUT1–3 and neuronal or astrocytic markers, both in an object-based manner using fluorescence line profiles, and in a pixel-based manner using dual-color scatter plots followed by the calculation of Pearson's correlation coefficient over all pixels with intensities significantly different from background. Our data provide no evidence in favor of the expression of any of the three VGLUTs by gray matter protoplasmic astrocytes of the primary somatosensory cortex, the thalamic ventrobasal nucleus, the hippocampus, and the cerebellum.

Introduction

The concept of the tripartite synapse, which includes thin astrocytic processes ensheathing the presynaptic terminals and the postsynaptic spines, has received considerable attention because it confers an active role in information processing to the electrically silent astrocytes (for review, see Araque et al., 1999). In this scheme, astrocytes, which express G-protein-coupled receptors,

respond to neuronal activity by Ca²⁺ elevations and the subsequent release of gliotransmitters that in turn regulates the neuronal excitability and synaptic transmission (Perea et al., 2009). However, the mechanisms of gliotransmission and their physiological and pathological relevance are still controversial (Fiacco et al., 2009; Hamilton and Attwell, 2010; Nedergaard and Verkhratsky, 2012).

At the neuronal synapse, fast glutamate release relies on small vesicles carrying one vesicular glutamate transporter (VGLUT) for glutamate vesicular storage (Fremeau et al., 2004a). VGLUT1 (Bellocchio et al., 2000; Takamori et al., 2000), VGLUT2 (Fremeau et al., 2001; Herzog et al., 2001), and to a lesser extent VGLUT3 (Ruel et al., 2008; Seal et al., 2008), are markers of glutamatergic synapses (Fremeau et al., 2004a; Moriyama and Yamamoto, 2004). VGLUT3, which is mainly expressed by cholinergic, serotonergic, and GABAergic neurons (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002; Herzog et al., 2004; Somogyi et al., 2004; Gillespie et al., 2005), may contribute to glutamate cotransmission (Trudeau and Gutierrez, 2007; Hnasko and Edwards, 2012) and facilitate the vesicular storage of cotransmitters (Gras et al., 2008). Interestingly all VGLUTs have been reported to be expressed by astrocytes, suggesting that astrocytes like neurons can release glutamate by exocytosis (Hamilton and Attwell, 2010). Evidence for VGLUT expression comes from cultured astrocytes (Anlauf and Derouiche, 2005; Montana et al., 2004; Zhang et al., 2004; Crippa et al., 2006; Bowser and Khakh, 2007; Stenovec et al., 2007; Marchaland et al., 2008; Ni and Parpura, 2009), as well as *in situ* studies (Fremeau

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Table 1. Antibodies used for fluorescence immunostaining and Western blotting

Experiments	Primary antibodies	Secondary antibodies ^a
Single staining		
VGLUT1. Cultured cortical astrocytes, Fig. 1D–E	Rabbit (Rb) BN3L2Bf VGLUT1 (Herzog et al., 2001), 1/2000	Alexa 488 or 555 Goat (G) anti Rb, 1/2000
Double staining		
VGLUT1 and S100 β . Cultured cortical astrocytes, Fig. 1B,C	Guinea pig (GP) VGLUT1, Millipore, Cat N° AB5905, 1/4000 and Rb S100 β Swant, 1/2000	Alexa 488 or 555 G anti GP, 1/2000 and Alexa 488 or 555 G anti Rb, 1/2000
VGLUT2 and GFAP. Cultured cortical astrocytes, Fig. 4B	GP VGLUT2, Millipore, Cat N° AB2251, 1/2000 and Mouse (M) GFAP, Millipore, MAB360, 1/1000	Alexa 633 G anti GP, 1/500 and Alexa 488 G anti M, 1/500
VGLUT3 and GFAP. Cultured cortical astrocytes, Fig. 5B	Rb VGLUT3, SYSY, Cat N° 135 203, 1/500 and M GFAP, Millipore, Cat N° MAB360, 1/2000	Alexa 555 G anti Rb, 1/2000 and Alexa 488 G anti M, 1/2000
GLT-1 and AQP4. Slices from C57Bl/6J mice, Fig. 6D	GP GLT-1, Frontier Science, Cat N° Af810, 1/500 and Rb AQP4, Sigma, Cat N° HPA014784, 1/500	Alexa 633 G anti GP, 1/500 and Alexa 488 G anti Rb, 1/500
VGLUT1 and synapsin. Slices from C57Bl/6J mice, Fig. 7	GP VGLUT1, Millipore, Cat N° AB5905, 1/4000 and Rb Synapsin, SYSY, Cat N° 106 002, 1/500	Alexa 633 G anti GP, 1/500 and Alexa 488 G anti Rb, 1/500
VGLUT1 and PSD95. Slices from C57Bl/6J mice, Fig. 7	GP VGLUT1, Millipore, Cat N° AB5905, 1/4000 and M PSD95, Abcam, Cat N° ab2723, 1/800	Alexa 633 G anti GP, 1/500 and Alexa 488 G anti M, 1/500
VGLUT2 and synapsin. Slices from C57Bl/6J mice, Fig. 10	GP VGLUT2, Millipore, Cat N° AB2251, 1/1000 and Rb Synapsin, SYSY, Cat N° 106 002, 1/500	Alexa 633 G anti GP, 1/500 and Alexa 488 G anti Rb, 1/500
VGLUT3 and GLT1. Slices from VGLUT3 KO mice, Fig. 5	Rb VGLUT3, SYSY, Cat N° 135 203, 1/500 and GP GLT-1, Frontier Science, Cat N° Af810, 1/500	Alexa 488 G anti Rb, 1/500 and Alexa 633 G anti GP, 1/500
Triple staining		
VGLUT1, synapsin and GFAP. Hippocampal astrocyte–neuron coculture Fig. 1A	GP VGLUT1, Millipore, Cat N° AB5905, 1/4000 and Rb Synapsin, SYSY, Cat N° 106 002, 1/500 and M GFAP, Millipore, Cat N° MAB360, 1/400	Alexa 633 G anti GP, 1/500 and Alexa 546 G anti Rb, 1/500 and Alexa 488 G anti M, 1/500
VGLUT2, synapsin and GFAP. Thalamic astrocyte–neuron co-culture Fig. 4A	GP VGLUT2, Millipore, Cat N° AB2251, 1/2000 and M GFAP, Millipore, Cat N° MAB360, 1/2000 or Rb GFAP, Abcam, Cat N° ab7260, 1/1000 and Rb synapsin, SYSY, Cat N° 106 002, 1/2000 or M synapsin 1, SYSY, Cat N° 106 001, 1/1000	Alexa 555 G anti GP, 1/2000 or Alexa 633 G anti GP 1/500 and Alexa 488 G anti M, 1/2000 or Alexa 546 G anti Rb 1/500 and Cy5 G anti Rb, 1/2000 or Alexa 488 G anti M 1/500
GFP, NeuN and S100 β . Slices from VGLUT2 eGFP mice, Fig. 3A	Chicken (Ch) GFP, Invitrogen, Cat N° A10262, 1/1000 and M NeuN, Millipore, Cat N° MAB377, 1/500 and Rb S100 β , Abcam, Cat N° ab52642, 1/500	Alexa 488 G anti Ch, 1/500 and Alexa 633 G anti M, 1/500 and Alexa 546 G anti Rb, 1/500
VGLUT1, S100 β and AQP4. Slices from C57Bl/6J mice, Fig. 6E, Fig. 7D and Fig. 8C,F	GP VGLUT1, Millipore, Cat N° AB5905, 1/4000 and Rb AQP4, Sigma, Cat N° HPA014784, 1/500 and M S100 β Abcam, Cat N° ab4066, 1/200	Alexa 633 G anti GP, 1/500 and Alexa 546 G anti Rb, 1/500 and Alexa 488 G anti M, 1/500
VGLUT1, NeuN and S100 β . Slices from C57Bl/6J mice, Figs. 7, 8	GP VGLUT1, Millipore, Cat N° AB5905, 1/4000 and M NeuN, Millipore, Cat N° MAB377, 1/500 and Rb S100 β Abcam, Cat N° ab52642, 1/500	Alexa 633 G anti GP, 1/500 and Alexa 488 G anti M, 1/500 and Alexa 546 G anti Rb, 1/500
VGLUT1, S100 β and GLT-1. Slices from C57Bl/6J mice, Figs. 6–8	GP VGLUT1, Millipore, Cat N° AB5905, 1/4000 and M S100 β Abcam, Cat N° ab4066, 1/200 and Rb GLT-1, Abcam, Cat N° ab41621, 1/500	Alexa 633 G anti GP, 1/500 and Alexa 488 G anti M, 1/500 and Alexa 546 G anti Rb, 1/500
VGLUT1 and S100 β . Slices from VGLUT1 ^{Venus} KI mice, with and without GFP antibody, Fig. 9	GP VGLUT1, Millipore, Cat N° AB5905, 1/4000 and Rb S100 β Abcam, Cat N° ab52642, 1/500 and Ch GFP, Invitrogen, Cat N° A10262, 1/1000	Alexa 633 G anti GP, 1/500 and Alexa 546 G anti Rb, 1/500 and Alexa 488 G anti Ch, 1/500
VGLUT1 and GLT-1. Slices from VGLUT1 ^{Venus} KI mice, with and without GFP antibody Fig. 9	GP VGLUT1, Millipore, Cat N° AB5905, 1/4000 and Rb GLT-1, Abcam, Cat N° ab41621, 1/500 and Ch GFP, Invitrogen, Cat N° A10262, 1/1000	Alexa 633 G anti GP, 1/500 and Alexa 546 G anti Rb, 1/500 and Alexa 488 G anti Ch, 1/500
VGLUT2, NeuN and S100 β . Slices from C57Bl/6J mice, Fig. 10	GP VGLUT2, Millipore, Cat N° AB2251, 1/1000 and M NeuN, Millipore, Cat N° MAB377, 1/500 and Rb S100 β Abcam, Cat N° ab52642, 1/500	Alexa 633 G anti GP, 1/500 and Alexa 488 G anti M, 1/500 and Alexa 546 G anti Rb, 1/500
VGLUT2, S100 β and GLT-1. Slices from C57Bl/6J mice, Figs. 6, 10	GP VGLUT2, Millipore, Cat N° AB2251, 1/1000 and M S100 β Abcam, Cat N° ab4066, 1/200 and Rb GLT-1, Abcam, Cat N° ab41621, 1/500	Alexa 633 G anti GP, 1/500 and Alexa 488 G anti M, 1/500 and Alexa 546 G anti Rb, 1/500
VGLUT3, S100 β and GLT-1. Slices from C57Bl/6J mice, Figs. 6, 11	Rb VGLUT3, SYSY, Cat N° 135 203, 1/500 and GP GLT-1, Frontier Science, Cat N° Af810, 1/500 and M S100 β Abcam, Cat N° ab4066, 1/200	Alexa 546 G anti Rb, 1/500 and Alexa 633 G anti GP, 1/500 and Alexa 488 G anti M, 1/500
Western blotting		
VGLUT1 Western blot, Fig. 1F	Rb BN3L2Bf VGLUT1 (Herzog et al., 2001), 1/5000	IRDye 800 G anti Rb (Rockland), 1/5000
VGLUT1 Western blot, Fig. 1G,H	GP VGLUT1, Millipore, Cat N° AB5905, 1/4000	IRDye 800 G anti Rb (Rockland), 1/5000
VGLUT2 Western blot, Fig. 4C	GP VGLUT2, Millipore, Cat N° AB2251, 1/5000	IRDye 800 G anti GP (Rockland), 1/5000
VGLUT3 Western blot, Fig. 5C	Rb VGLUT3, SYSY, Cat N° 135 203, 1/2000	IRDye 800 G anti Rb (Rockland), 1/5000

^aAll secondary antibodies for immunostaining are from Invitrogen.

Table 2. Combinations of excitation wavelengths and filters

Fluorophore	ex (nm)	DC (nm)	em (nm)
Confocal microscopy*			
Alexa 488	488	AOBS	BP498-550
Alexa 488	488	HFT/488/543/633	BP505-530
Alexa 546	543	HFT/488/543/633	BP560-615
Alexa 555	561	AOBS	BP569-631
Alexa 633	633	HFT/488/543/633	650LP
Cy5	633	AOBS	BP643-720
TIRFM			
GFP and FM4-64	488	PolyX [†]	535(50) and 675(50)BP [‡]
Venus and FM4-64	488	PolyX [†]	535(50) and 675(50)BP [‡]
Epifluorescence			
Spectral imaging of VGLUT1 ^{Venus} brain slice	458**	500LP	510(20)-535(50)-575(50)-600(40)-670(50)BP
Spectral imaging of cultured astrocytes	458 ⁺	500LP	510(20)-535(50)-560(40)-615(45)-670(40)BP

*Acousto-optical beam splitter (AOBS) was used with Leica SP5 confocal microscope and other dichroics with Zeiss LSM 510 confocal microscope. **Center wavelength of the 18 nm (full-width at half-maximum) excitation band of a TILL polychrome II tunable light source. [†]PolyX, custom dual dichroic mirror with 488/568/NIR reflection bands and low ripple high-transmission elsewhere (AHF Analysentechnik). [‡]Denotes filters for simultaneous view with a custom dual-view device. DC, dichroic; ex, excitation wavelength; em, emission filter; TIRFM, total internal reflection fluorescence microscopy.

et al., 2002; Bezzi et al., 2004; Platel et al., 2010; Bergersen et al., 2012; Ormel et al., 2012b). Evidence is largely based on immunostaining and remains controversial because other authors failed to confirm astrocytic VGLUT expression (Hayashi et al., 2001; Graziano et al., 2008; Restani et al., 2011), raising the possibility that immunostaining might be compromised by nonspecific labeling (Fritschy, 2008). This concern has been fueled by the observation that, unlike synaptic glutamate release, glutamate release from astrocytes is not affected by a VGLUT-dependent effect of acetoacetate (Juge et al., 2010). Furthermore, a transcriptome analysis was unable to detect the VGLUT1 and VGLUT2 mRNAs in astrocytes (Cahoy et al., 2008). Finally, our previous study shows that anion channels rather than vesicular exocytosis mediate glutamate release by astrocytes in culture (Li et al., 2012). Hence, we decided to reexamine the expression of VGLUT1–3 proteins in astrocytes, both in culture and *in situ*.

We studied the VGLUT1–3 protein expression at the single vesicle level in cultured astrocytes using total internal reflection fluorescence microscopy (TIRFM), and at the cellular level in the cortical, hippocampal, and cerebellar cortex of fixed brain slices using confocal microscopy. Control experiments using immunostaining and VGLUT1–3 knock-out (KO) mice were performed. The expression of VGLUT1–2 in astrocytes was also investigated using VGLUT1^{Venus} knock-in (KI) (Herzog et al., 2011), and VGLUT2-EGFP transgenic (Tg) mouse lines.

Materials and Methods

All experiments followed the European Union and institutional guidelines for the care and use of laboratory animals (Council directive 86/609EEC). Previous work has described the VGLUT1 KO (Wojcik et al., 2004), VGLUT2 KO (Moechars et al., 2006), VGLUT3 KO (Gras et al., 2008), and VGLUT1^{Venus} KI (Herzog et al., 2011) mouse lines. The Bac Tg VGLUT2-EGFP mouse line [Tg(Slc17a6-EGFP)FY115Gsat #11835-UCD] was provided by the Mutant Mouse Regional Resource Center (Gong et al., 2003). The colony was maintained locally by crossing the heterozygous (HZ) mice with wild-type (WT) NMRI mice. Combinations of the primary and secondary antibodies used for fluorescence immunostaining and Western blotting are listed in Table 1. The combinations of excitation wavelengths (ex), dichroic (DC), and emission filters (em) used are listed in Table 2.

Cell preparation and immunofluorescence. Cortical or hippocampal astrocytes were prepared from newborn P0–P1 (P0 being the day of birth) NMRI mice of either sex, embryonic E18 (E0 being the day of mating) VGLUT2 KO of either sex, and P0 VGLUT1 KO and P0 VGLUT3 KO mice of either sex as previously described (Li et al., 2009). Briefly, the neocortex or the hippocampus was dissected and mechanically dissociated. Cells were plated and maintained in Petri dishes during 1 week to

reach confluence before their transfer onto coverslips (#1, BK-7, 25 mm diameter, Marienfeld Superior, Thermo Fisher Menzel-Gläser). Coverslips (12 mm diameter) were used for immunostainings. Cells were plated in plastic Petri dishes for Western blot analysis. Cultured astrocytes were maintained 10–15 d *in vitro* (DIV) before fixation. Cortical and thalamic astrocyte/neuron cocultures were isolated from E16–E17 VGLUT2 KO mice of either sex. Hippocampal astrocyte/neuron cocultures were prepared from P0 VGLUT1 KO mice of either sex. Cells were seeded on poly-D-lysine-treated coverslips. Thalamic cocultures were maintained in a medium containing DMEM-F12 supplied with 5% fetal bovine serum (FBS), 1.3% D-glucose 45%, serum extender (1/1000), and penicillin/streptomycin (1/500). Cortical and hippocampal cocultures were maintained in a minimum essential medium with 10% FBS, 1% L-glutamine, 1.3% D-glucose 45%, 1% sodium pyruvate, 2% B27, and penicillin/streptomycin (1/1000). Cells were maintained in half of this serum and half of medium of primary astrocytes containing growth factors for neurons. Astrocyte/neuron cocultures were maintained 10–15 DIV before fixation. Cell media and supplements were from Invitrogen.

For immunofluorescence, astrocytes and astrocyte/neuron cocultures were treated with 4% paraformaldehyde (PFA; EMS 15710; Electron Microscopy Services) for 10 min at room temperature (RT; 22–23°C). After permeabilization and blockage of unspecific sites with PBS 1X (Sigma, P4417), 1% Triton X-100, and 4% normal goat serum (NGS; 1 h, RT; Gibco, 16210-064), the cells were probed with the respective primary antibodies in the same solution overnight at 4°C. After being washed with PBS 1X three times at RT, cells were incubated with secondary antibodies (2 h, RT). The same fluorophore combinations were used in all experiments to allow for direct comparison and exclude detection bias (Table 1). After three final washes (PBS, 10 min, RT), cells were mounted with Mowiol 4–88 (Calbiochem) onto microscope slides. Antibody specificity was controlled for by omitting the primary antibody and by using KO mice when available. Triple-immunofluorescence images of VGLUT1, synapsin, and glial fibrillary acidic protein (GFAP) in hippocampal astrocyte/neuron cocultures were acquired using a confocal laser-scanning microscope (Axiovert LSM 510; Carl Zeiss) equipped with Ar⁺ (488, 543 nm) and HeNe (633 nm) gas lasers and multi-alkali side-on meshless photomultiplier tubes (PMTs; R6357; Hamamatsu), using a ×63/NA 1.4 Plan-Neofluar oil objective. Some images of cultured cells (as indicated in the figure legends) were acquired with another confocal laser-scanning microscope (Leica SP5 Microsystems) using a ×63/NA 1.32 PL APO oil objective.

Immunofluorescence of slice preparations. Adult (8 weeks) C57BL/6J WT of either sex, male VGLUT2-EGFP, VGLUT3 KO of either sex, and male VGLUT1^{Venus} KI mice were anesthetized by intraperitoneal injection of pentobarbital (20 mg/kg). Animals were perfused with intracardiac PBS 1X to remove blood cells and subsequently with 4% PFA for fixation. The brains were removed and postfixed overnight at 4°C in 4% PFA. After rinsing in PBS 1X, the brains were conserved in PBS 1X. Thin

Figure 1. Astrocytes in culture do not express VGLUT1. **A**, Left, Confocal images of hippocampal astrocyte/neuron cocultures prepared from VGLUT1 HZ and KO mice and immunolabeled with antibodies against VGLUT1, synapsin, and GFAP. VGLUT1 fluorescence was seen in HZ but not in KO preparations. Right, The neuronal VGLUT1 fluorescence, quantified as (Figure legend continues.)

(45 μm) coronal slices were cut using a vibratome (VT1000S; Leica). After permeabilization with 1% Triton X-100 (Sigma, T9284) and blockade of the unspecific sites with 4% NGS (1 h, RT), slices were stained with primary antibodies in a PBS 1X solution with 0.2% Triton X-100 and 2% NGS for overnight at 4°C. Slices were then washed with PBS three times at RT and incubated with secondary antibodies (PBS 1X, 2% NGS, 1 h, RT). After three final washes (PBS 1X, 10 min at RT) the slices were mounted in Mowiol 4–88 onto microscope slides and sealed with coverslips. Fluorescence images were collected with a Zeiss LSM 510 confocal microscope. Overviews were taken with $\times 10/\text{NA } 0.3$ or $\times 20/\text{NA } 0.5$ air objectives. Single confocal sections taken with the $\times 63/\text{NA } 1.4$ Plan-NeoFluar oil-immersion objective were used for colocalization analysis (see below). The confocal pinhole was systematically set to 0.8 Airy units to maximize axial resolution rather than the signal intensity. Sequential acquisitions were made to minimize cross-excitation. For each channel, the laser power, offset, and PMT gain were adjusted using a cold/gray-level/hot look-up table to avoid under exposure and saturation. Levels of autofluorescence and detected back-scattered excitation light were found negligible at the laser powers used for acquisition (typically $<10\%$ power of the 30 mW available at 488 nm and 5 mW available at 633 nm, and $<60\%$ of the 1 mW of the 543 nm line, respectively).

Western blotting. Western blotting was performed on pure astrocyte cultures, primary neuron cultures, and tissue homogenates of VGLUT1-expressing regions of the mouse brain of either sex with 5 μg of protein loaded per lane. Astrocytes were cultured from two independent litters of either sex from each mouse line and lysed 2 weeks after culturing. Samples were loaded on precast NuPAGE Novex 10% Bis-Tris Midi Gels (WG1202BOX; Invitrogen), migrated in NuPAGE MES-SDS Running Buffer (NP0002; Invitrogen), and transferred to nitrocellulose membranes (LC2009; Invitrogen) in Tris-borate buffer. IRDye 680/800 coupled secondary antibodies were used for semiquantitative detection and blots were exposed to reveal all signals, including background bands. Membranes were scanned using Odyssey infrared imaging system (LI-COR). Ponceau S staining was used for assessing the correct loading of samples as the heterogeneity in protein sources prevented us from using internal loading controls with confidence.

Combined multispectral epifluorescence and TIRF live-cell imaging. A custom-built inverted microscope (Nadrigny et al., 2006; Li et al., 2008) was used for bright-field polychromatic epifluorescence (EPI) imaging and through-the-objective TIRF imaging using a PlanApo TIRF $\times 60/\text{NA } 1.45$ oil objective (Olympus). A Polychrome II light source (TILL Photonics) provided tunable narrowband (18 nm full-width at half-maximum) EPI illumination. The 488 and 568 nm lines used for TIRFM were isolated from the beam of an Ar^+/Kr^+ multiline gas laser (CVI; Melles Griot) with an acousto-optical tunable filter (AA.Opt) and directed onto the glass/water interface at a supercritical angle. We estimated the effective penetration depth of the order of 200 nm (Nadrigny et al., 2007). Fluorescence images were further magnified ($\times 2$) and projected on an electron multiplying charge-coupled device (EMCCD; QuantEM 512; Princeton Instruments). All devices were controlled by MetaMorph 7.0. The effective pixel size in the sample plane was 133 nm.

←

(Figure legend continued.) the ratio of VGLUT1/synapsin, was significantly smaller in VGLUT1 KO than HZ cell preparations. The VGLUT1 fluorescence of astrocytes, quantified as the ratio of VGLUT1/GFAP, was weak and similar in VGLUT1 KO and HZ cell preparations. **B**, Confocal images of pure cortical astrocytes in culture immunolabeled with antibodies against VGLUT1 and S100 β . The VGLUT1 fluorescence was similar for astrocytes prepared from VGLUT1 KO and WT mice. Swapping the secondary antibodies (**C**), using another VGLUT1 primary antibody (**D**), and swapping again the secondary antibodies (**E**) did not change the results (see Table 1). **F**, Western blot analysis of VGLUT1 expression by pure cultured cortical astrocytes from WT and VGLUT1 KO and HZ mice. Control VGLUT1 signal was obtained from brainstem and cortical tissue (Cx). Correct loading of samples is shown in the Ponceau S staining (below). Although we can detect weak VGLUT1 expression from brainstem tissue, we were not able to detect any specific signal from the astrocyte cultures (above). **G**, Lack of specific VGLUT1 band found for pure astrocyte cultures by Western blot with a different primary antibody (Millipore). Hipp, hippocampal culture; Hom, whole-brain homogenate. **H**, Western blot analysis of VGLUT1 expression in pure astrocyte cultures maintained 10, 17, and 24 DIV. Scale bars, 20 μm .

A custom image splitter permitted the simultaneous acquisition of dual-channel fluorescence on the same EMCCD camera chip. For emission spectral imaging, five emission spectral images were acquired upon 458 nm EPI excitation sequentially through narrow bandpass emission filters housed in a motorized filter wheel (details in Table 2).

Astrocytes were imaged 2–6 d after their transfer into secondary culture. All cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. VGLUT1-Venus (1.4 $\mu\text{g}/\mu\text{l}$) was transfected into cultured astrocytes using Lipofectamine 2000 (Invitrogen) following standard protocols. During imaging at RT, the cells were constantly perfused at 0.5–1 ml/min with extracellular solution containing the following (in mM): 140 NaCl, 5.5 KCl, 1.8 CaCl_2 , 1 MgCl_2 , 20 glucose, and 10 HEPES, pH 7.3 was adjusted with NaOH. Astrocytic lysosomes were labeled with the red-fluorescent styryl pyridinium dye FM4–64 (Invitrogen; 6.7 μM , 15 min), and rinsed for 30 min with dye-free solution before imaging (Li et al., 2008).

Colocalization analysis. We first assessed colocalization among different fluorescent markers using morphological landmarks, by drawing line profiles across morphologically identified neuronal and astrocytic processes. In addition to this object-based colocalization analysis, we systematically calculated pixel-based descriptors, incorporating the intensity information of all image pixels that had an intensity significantly different from background (see below). An intuitive way to see if a given pixel carries both fluorophores is a scatter plot in which the normalized intensity F/F_{max} is graphed pixelwise in a 2D histogram. Pixels in which both dyes are detected at the same F/F_{max} lay on a straight line from the origin to (1,1). Image noise or variable labeling ratios add noise to this pattern, but points are still symmetrically spread about the 45° line. However, albeit commonly used, these plots are difficult to interpret, because pixels with similar fluorophore ratios superimpose and obstruct each other when analyzing large images. Thus, the graphical impression of such pixel-dense scatter plots can be misleading. We therefore developed a density-encoded scatter plot in which the pixel abundance at a given intensity ($F_1/F_{1,\text{max}}(i)$, $F_2/F_{2,\text{max}}(i)$) is pseudocolor encoded from blue (few pixels) to red (many pixels). Thus, in addition to representing intensity ratios, this density-encoded scatter permits the visualization of the information contained in the ensemble of pixels. We also systematically measured the mean and standard deviation (SD) of the background signal and identified intensity regions on the scatter plot that contain intensity data significantly different from background (mean + 3 SD).

To compare fluorescent images, we calculated Pearson's correlation coefficient r_{12} as follows:

$$r_{12} = \frac{\sum_i (F_1(i) - \langle F_1 \rangle_{\forall i})(F_2(i) - \langle F_2 \rangle_{\forall i})}{\sqrt{\sum_i (F_1(i) - \langle F_1 \rangle_{\forall i})^2 \cdot \sum_i (F_2(i) - \langle F_2 \rangle_{\forall i})^2}} \quad (1)$$

where $F(i)$ represents the fluorescence intensity of each pixel i and F_1 and F_2 the average over all N pixels of the component images 1 and 2, respectively. N is the total number of pixels in each image. This method has the advantage of taking into account both the image background and the specific labeling, and allowing a comparison of images with different average signal intensities. In fact, r_{12} measures, for both color channels, the excursion of the pixel intensity from the mean signal. Background intensities were determined on the same image from at least five small regions of interest (ROIs; ranging from $2 \times 2 \mu\text{m}$ to $5 \times 5 \mu\text{m}$) devoid of fluorescent objects, e.g., outside the cell contour in culture, or within unlabeled cell nuclei in slices. Colocalization parameters were calculated from pixels the intensity of which exceeded the mean of the background by three times its SD. This intensity limit is shown as horizontal and vertical lines on the scatter plots (see Figs. 7B, 10C). The meaningful upper (i.e., positive control, +CTR) and lower bound (negative control, –CTR) of r_{12} values (Li et al., 2008) were determined by performing dual-color immunostaining with antibodies against two presynaptic proteins, VGLUT1 and synapsin (Huttner et al., 1983), and against VGLUT1 and PSD-95, a postsynaptic density scaffolding protein selectively expressed by the excitatory synapses (Hunt et al., 1996; Heller et al., 2012), respectively.

To further demonstrate the significance of our colocalization analysis, we artificially introduced a variable pixel shift between images acquired in dif-

Figure 2. Cultured astrocytes from VGLUT1^{Venus} KI mice do not express Venus. **A**, Top, Autoscaled EPI images of an acute live hippocampal slice prepared from a VGLUT1^{Venus} KI mouse. Images were sequentially acquired using discrete narrowband emission filters (numbers indicate the filter central wavelength; details in Table 2). Stratum radiatum (SR) and stratum lucidum (SL) CA3 regions appeared more fluorescent. Bottom, Five-point spectra of VGLUT1-Venus KI CA3 ROIs measured in SR (black trace, average of eight $50 \times 50 \mu\text{m}$ ROIs from two slices; the black circle in **A** at 535 nm shows one SR ROI) and in a nonfluorescent region to estimate the background (gray trace, average of six ROIs; gray circle shows one background ROI). Scale bar, $100 \mu\text{m}$. **B**, Top, Dual-color TIRFM images of a live cultured astrocyte prepared from VGLUT1^{Venus} KI mice and loaded with the red-fluorescent lysosomal marker FM4-64 (spectrally distinct from the yellow fluorescent Venus) to identify the focal plane. Inset, Cultured live astrocyte transfected with a VGLUT1-Venus-encoding plasmid expressed highly fluorescent vesicles. Scale bar, $10 \mu\text{m}$. Bottom, Intensity and density of fluorescent puncta in live astrocytes from VGLUT1^{Venus} KI mice labeled with FM4-64 in dual-color TIRFM images (VGLUT1-Venus and FM4-64) and in live astrocytes transfected with VGLUT1-Venus (Transfection). **C**, Spectral profile of the yellow fluorescence measured from individual vesicles in live astrocytes transfected with VGLUT1-Venus ($n = 10$ vesicles from 2 cells). Scale bar, $1 \mu\text{m}$. **D**, Spectral profile of weakly yellow fluorescent puncta seen in live astrocytes prepared from VGLUT1^{Venus} KI mice (ROI shown by white arrow in **B**, $n = 17$ ROIs from four cells). Scale bar, $1 \mu\text{m}$. **E**, Spectral profile of autofluorescence of live astrocytes prepared from WT littermates ($n = 14$ ROIs from 4 cells).

ferent color channels and recalculated r_{12} for each shifted pair. This analysis was performed in all four cardinal directions, and the obtained r_{12} values averaged and plotted against the offset. Thus, if the fluorescent images of two proteins overlap, r_{12} will decrease rapidly on a length-scale characteristic of the size of the object that carries both fluorophores. Fluorophore presence in juxtaposed but distinct structures is manifest as a maximum of r_{12} at a pixel value different from zero. Absence of colocalization or a low signal-to-noise ratio will appear as a negligible modulation of r_{12} with pixel shift.

Image analysis and spectral unmixing. To count the density of fluorescent puncta, we first thresholded the image using a systematic 10% intensity criterion (fraction of the peak intensity after background subtraction), segmented the image with a watershed particle analyzing process using National Institutes of Health ImageJ and MetaMorph 7.0,

and then counted the number of puncta/ μm^2 . Objects had to be smaller than 8×8 pixels and larger than 2×2 pixels. Their number was normalized with the total area of the ROI. For TIRFM images, the contour of the cellular footprint was traced by hand and the mean fluorescence intensity within this ROI was measured.

We compared the fluorescence spectra of individual diffraction-limited puncta (putative vesicles) in astrocytes transfected with VGLUT1-Venus, and in astrocytes from the VGLUT1^{Venus} KI mice as well as their WT littermates, by spectral imaging and linear unmixing (Nadrigny et al., 2006, 2007). Briefly, all fluorophore spectra were determined by acquiring five discrete EPI images using narrowband emission filters (Table 2). We subtracted from the raw images the background taken from cell-free regions, and normalized the five-point spectra to the total fluorescence intensity (equal energy). The

normalized five-point spectra were then compared with that of autofluorescence or cytoplasmically expressed Venus, by calculating their spectral angle Θ_λ

$$\Theta_\lambda = \arccos\left(\frac{\hat{\mathbf{R}}(x,y) \bullet \hat{\mathbf{S}}(x,y)}{\|\hat{\mathbf{R}}(x,y)\| \cdot \|\hat{\mathbf{S}}(x,y)\|}\right), \quad (2)$$

where $\|\mathbf{x}\| = \sqrt{\sum_i x_i^2}$. The average spectra of two species were considered different when the spectral angle was bigger than the inner species variability of the respective vector bundles.

Statistics. All normally distributed data are expressed as mean $\pm$ SD, and the *t* test was used for testing the significance of *p* values. Non-normally distributed data were compared using their median $\pm$ absolute deviation and nonparametric tests (Kolmogorov–Smirnov). All statistics used MATLAB (MathWorks). *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant.

Results

Evidence for VGLUT1 and VGLUT2 expression by astrocytes in culture has been provided using immunostaining (Bezzi et al., 2004; Montana et al., 2004; Zhang et al., 2004; Anlauf and Derouiche, 2005; Bowser and Khakh, 2007; Stenovec et al., 2007; Marchaland et al., 2008); however, control experiments with KO mice to examine the specificity of antibodies were performed in only two cases (Ormel et al., 2012a,b). As a first step, we therefore systematically re-investigated VGLUT1–3 immunostaining in cultured astrocytes from WT, HZ, and KO littermates, and studied the expression of fluorescent protein by cultured astrocytes from VGLUT1^{Venus} KI and VGLUT2-EGFP mice.

VGLUT1 expression in cultured astrocytes

VGLUT1 is widely expressed by hippocampal and neocortical neurons (Fremeau et al., 2004a). There is also some evidence for VGLUT1 expression by cultured astrocytes (Montana et al., 2004; Zhang et al., 2004; Anlauf and Derouiche, 2005; Bowser and Khakh, 2007; Stenovec et al., 2007; Marchaland et al., 2008); however, controls using VGLUT1 KO mice have not been performed. We first tested the possible expression of VGLUT1 in astrocytes using hippocampal neuron/astrocyte cocultures and pure cultures of cortical astrocytes (Fig. 1). Experiments were performed using hippocampal neuron/astrocyte cocultures prepared from VGLUT1 HZ and KO mice and triple immunostaining with antibodies against VGLUT1, synapsin (a presynaptic neuronal protein), and the astrocyte-specific GFAP, respectively. As expected, the neuronal VGLUT1 labeling was lost in cultures from VGLUT1 KO mice, confirming the neuronal expression of VGLUT1. The VGLUT1 fluores-

Figure 3. Astrocytes from VGLUT2-EGFP mice do not express EGFP. **A**, Top, Triple-color confocal images in thalamic VB nucleus of a VGLUT2-EGFP Tg mouse immunostained with antibodies against NeuN, S100β, and GFP ($\times 63$ NA1.4 oil objective). Bottom, Line profiles across somatic regions (between the arrowheads) show that cell bodies of S100β-positive cells, unlike NeuN-positive relay neurons, do not express EGFP. Scale bar, 20 μ m. **B**, Left, Dual-color TIRFM images of cultured astrocytes prepared from VGLUT2-EGFP Tg mice and their WT littermates and maintained 13 DIV. Cells were colabeled with the red-fluorescent lysosomal marker FM4–64. No difference in EGFP signal was found between astrocytes prepared from VGLUT2-EGFP and WT mice. Similar results were obtained when astrocytes were kept in culture for 26 DIV (**C**). Scale bars: 10 μ m.

cence, quantified as the VGLUT1/synapsin fluorescence ratio, was significantly reduced in neurons from VGLUT1 KO (0.05 ± 0.09 , *n* = 533 puncta, 12 cells) compared with HZ (0.25 ± 0.1 , *n* = 766 puncta, 9 cells; *p* < 0.001) mice. In contrast, the weak VGLUT1 fluorescence seen in GFAP-positive astrocytes, quantified as VGLUT1/GFAP fluorescence ratio, was similar in cells from VGLUT1 HZ (0.28 ± 0.12 , *n* = 70 cells) and KO mice (0.28 ± 0.16 , *n* = 87 cells; *p* = 0.87), indicating that the fluorescence detected at the power and gain levels required to reveal VGLUT1 signal is, in

Figure 4. Astrocytes in culture do not express a specific VGLUT2 signal. **A**, Thalamic astrocyte/neuron cocultures were prepared from VGLUT2 WT, HZ, and KO mice and immunostained with antibodies against VGLUT2, synapsin, and GFAP. The VGLUT2 immunostaining seen in synapsin-positive neurons from VGLUT2 WT ($n = 500$ synapsin-positive puncta from 6 trials) and HZ ($n = 881$ puncta from 8 trials) mice was absent in neurons from VGLUT2 KO mice ($n = 635$ and 710 puncta, respectively; $p < 0.001$). The weak VGLUT2 signal seen in GFAP-positive astrocytes was undistinguishable in WT ($n = 58$ cells), HZ ($n = 48$ cells), and KO ($n = 72$ and 56 cells, respectively) preparations. **B**, Pure cortical astrocytic cultures were immunostained with VGLUT2 and GFAP antibodies. The VGLUT2 labeling in astrocytes, albeit detectable, shows no difference between WT, HZ, and KO mice. **C**, Western blot of VGLUT2 protein in pure cultured cortical astrocytes (WT, HZ, KO), pure cultured hippocampal neurons (Hipp. culture), and brain homogenate (Brain-homog.) from WT animals. Left, Western blots of VGLUT2 show a strong specific labeling in WT brain homogenate, a weak signal in hippocampal neurons, and no specific signal in WT or HZ astrocytes compared with KO cells from littermates. Right, Ponceau S staining indicates a comparable amount of protein loaded in all samples. Scale bars: $20 \mu\text{m}$.

fact, not specific (Fig. 1A). Cortical astrocytes in culture were also immunostained with antibodies against VGLUT1 and S100 β (a cytosolic Ca^{2+} binding protein expressed by most cortical astrocytes; Vives et al., 2003). The VGLUT1 and S100 β fluorescence intensity, the VGLUT1/S100 β fluorescence ratio, and the density of the VGLUT1-positive puncta were undistinguishable in astrocytes from

VGLUT1 KO and HZ mice (Fig. 1B). Similar results were obtained using different secondary (Fig. 1C), primary VGLUT1 (Fig. 1D), and again swapping the secondary (Fig. 1E) antibodies. Finally, Western blots failed to detect any specific VGLUT1 band in pure cultured astrocytes from WT mice, as compared with VGLUT1 KO preparations (Fig. 1F, G). A strong band at the correct molecular weight was

detected in the WT but not in the KO cortical homogenate (Fig. 1*G,H*) and a weak but clearly visible band was present in the brainstem, a structure receiving very few VGLUT1 afferents (Herzog et al., 2001). Ponceau S staining of the membrane indicates nearly equal loading of all samples. Distinct band profiles of the Ponceau S stainings reflect different protein compositions between the brain homogenates and the astrocyte cultures (Fig. 1*F–H*). Similar results were obtained from two different antibodies (Fig. 1*F*; rabbit anti-VGLUT1 BN 3L2BF from Herzog et al., 2001 and Fig. 1*G*; guinea pig AB5905 from Millipore; see also Table 1). In addition, we observed consistent absence of VGLUT1 signal in astrocytes from WT and KO littermates kept for 10, 17, and 24 DIV (Fig. 1*H*). These results indicate that cultured astrocytes do not express significant levels of VGLUT1 protein.

An alternative strategy to using immunofluorescence is based on the use of fluorescent KI mice with the fluorescent Venus DNA inserted in frame before the stop codon of VGLUT1 genomic locus. Hence VGLUT1^{Venus} is expressed in place of the endogenous protein virtually without any alterations. As a result, Venus fluorescence is enriched in the axon synaptic terminals of the VGLUT1-positive neurons (Herzog et al., 2011). We used this mouse line as an independent assay for testing the possible VGLUT1 expression by cortical astrocytes in culture. We first confirmed in acute brain slices the presence of a strong fluorescent signal in hippocampal layers, which are enriched in VGLUT1 with a fluorescence emission peak near 528 nm, as expected (Herzog et al., 2011) for the yellow-fluorescent Venus protein (Fig. 2*A*). Live-cell dual-color TIRFM did not reveal a significant amount of Venus in cultured cortical astrocytes prepared from VGLUT1^{Venus} KI mice (Fig. 2*B*). This was not due to the limited sensitivity of our microscope or to focusing error, because individual near-membrane lysosomes labeled with the red styryl dye FM4-64 (Zhang et al., 2007; Li et al., 2008) were reliably detected (whole-cell fluorescence, 25.6 ± 7.9 a.u., $n = 8$ cells for FM4-64 vs 1.9 ± 0.9 a.u., $n = 14$ cells for Venus). Likewise, astrocytes transfected with a VGLUT1-Venus plasmid expressed a high density ($0.1 \pm 0.03/\mu\text{m}^2$) of highly fluorescent puncta (67.4 ± 30.2 a.u., $n = 13$ cells), demonstrating our ability to detect individual Venus-positive fluorescent puncta. Furthermore, the spectral profile of individual fluorescent vesicles in the VGLUT1-Venus-transfected astrocytes (Fig. 2*C*) differed from that of the weakly fluorescent puncta in cultured astrocytes prepared from VGLUT1^{Venus} KI mice (Fig. 2*D*), the latter being similar to the spectral profile of autofluorescence (AF) seen in astrocytes prepared from WT mice (Fig. 2*E*).

Figure 5. Astrocytes do not express a specific VGLUT3 signal. **A**, Dual-color confocal images of CA1 hippocampal regions in the VGLUT3 KO mice and their WT littermates colabeled with antibodies against VGLUT3 and GLT-1. The density and intensity of the VGLUT3-positive fluorescent puncta were quantified, as well as the intensity of the GLT-1 staining. The low-density VGLUT3-positive puncta seen in the SP and SR of WT littermates (WT) disappeared in the slices of the VGLUT3 KO mice (KO) without any change of the GLT-1 fluorescent signal. **B**, Pure cortical astrocyte cultures from WT and VGLUT3 KO mice were coimmunostained with antibodies against VGLUT3 and GFAP. The weak VGLUT3 signal, quantified as VGLUT3/GFAP ratio, was similar in astrocytes from WT and VGLUT3 KO mice. **C**, Western blot analysis of cultured cortical astrocytes and striatal tissue from VGLUT3 WT, HZ, and KO mice. Ponceau S staining shows that an equal amount of protein was loaded in all lanes (right). A specific signal is present only in the WT striatum sample, while missing from KO striatum as well as astrocytes of all genotypes (left). Scale bars: 20 μm .

Similarity among spectral profiles was quantified using the inner-species and interspecies variability of the spectral angle (see Materials and Methods). The innerspecies variability of the spectral angle was smaller (8.4 and 6.21° for VGLUT1-Venus-transfected astrocytes, and in astrocytes prepared from VGLUT1^{Venus} KI mice, respectively) than the interspecies spectral angle between these two signals (31.7°), meaning that the spectral vector bundles are distinct and that the faint signal seen in the Venus channel in astrocytes prepared from VGLUT1^{Venus} KI mice originated from a fluorophore different from Venus. Rather, it appears to be due to AF as evidenced by the small interspecies variability of the spectral angle between the faint Venus and AF signals, which is 4.01° , a value smaller than the innerspecies variability of AF spec-

Figure 6. Colocalization of the astrocytic markers. **A**, High-magnification ($\times 63$ /NA 1.4 oil objective) confocal images in cerebellar stratum molecular (SM). Fixed slices were coimmunostained with antibodies against S100 β (green) and GLT-1 (red). The line profiles between the arrowheads of the magnified subregion ($14 \times 14 \mu\text{m}$) suggest a colocalization between S100 β and GLT-1. Scale bar, $20 \mu\text{m}$. **B**, Similar line profiles in S1 cortex colabeled with antibodies against S100 β and GLT-1 suggest also a colocalization between S100 β and GLT-1. Scale bar, $5 \mu\text{m}$. **C**, Pearson's correlation coefficient r_{12} values for S100 β and GLT-1 in layers 2–6 S1 cortex (Cx), in the SR of the CA1 and CA3 hippocampal regions (CA1 and CA3), and in cerebellar SM (Cb). The number of regions analyzed is given in parenthesis. The positive control (+CTR), shown by the gray dashed line, is the average r_{12} value for VGLUT1 and synapsin calculated in the same regions. The parameter was similar in all regions ($p > 0.13$). Right, The r_{12} values plotted against the pixel shift show an immediate and significant decrease further validating the spatial colocalization of S100 β and GLT-1 in astrocytic profiles. Slices were taken from two animals for each condition. **D**, **E**, Colocalization between astrocytic membrane markers AQ4 and GLT-1 (**D**), and between AQ4 and S100 β (**E**). The representative confocal image was taken from the hippocampal CA3 region (**D**) and cortex (**E**), respectively. Colocalization analysis was performed for cortex (Cx); hippocampus (Hip) including CA1, CA3, and fascia dentate region; and cerebellum (Cb) including SM and GL layer. Scale bars: $20 \mu\text{m}$.

Figure 7. VGLUT1 and astrocytic markers do not colocalize in the somatosensory S1 cortex. **A**, Left, High-magnification ($\times 63/\text{NA } 1.4$ oil objective) confocal images of S1 cortical slices immunostained with antibodies against VGLUT1; synapsin as a positive control; PSD-95 as a negative control; and astrocytic markers S100 β , GLT-1, and AQ4. Middle, Magnified ROIs indicated by a white square on the left side. Right, Fluorescence intensity line profiles between the white arrows shown in the magnified subregions. Scale bar, 20 μm . **B**, (Figure legend continues.)

tra, which was 6.63° . In line with the immunofluorescence data, our results indicate that cortical astrocytes cultured from VGLUT1^{Venus} KI mice do not express detectable amounts of the VGLUT1 protein.

VGLUT2 expression in cultured astrocytes

VGLUT1 and VGLUT2 are both expressed by glutamatergic neurons; however, they are found in different subtypes of neurons (Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001; Sakata-Haga et al., 2001; Takamori et al., 2001), VGLUT1 being expressed by most cortical neurons, and VGLUT2 by the thalamocortical relay neurons (Fremeau et al., 2004b; Nakamura et al., 2005). Previous studies found VGLUT2 expression in cultured astrocytes (Montana et al., 2004; Anlauf and Derouiche, 2005; Crippa et al., 2006; Ni and Parpura, 2009); however, control experiments using VGLUT2 KO mice are missing. We therefore investigated the possible expression of VGLUT2 by cultured astrocytes prepared from VGLUT2-EGFP Tg mice, VGLUT2 KO mice, and their WT/HZ/KO littermates.

Unlike in the VGLUT1^{Venus} KI mice used above, EGFP is not targeted to VGLUT2-expressing vesicles in VGLUT2-EGFP Tg mice and it labels the cytosol of VGLUT2-expressing cells. In fixed thalamic slices, cell bodies of NeuN-positive ventrobasal (VB) neurons were labeled with EGFP (Fig. 3A), as expected for VGLUT2-expressing relay neurons (Fremeau et al., 2004b; Nakamura et al., 2005). Interestingly, in the same slices, the cell bodies of S100 β -positive astrocytes lacked detectable amounts of EGFP. Next, to examine a possible glial expression of VGLUT2 in culture, astrocytes from VGLUT2-EGFP HZ and WT littermates were maintained 13 and 26 DIV. Using live-cell dual-color EGFP and FM4–64 TIRF imaging (Li et al., 2008), we found no significant difference in the EGFP signal between the two types of astrocyte preparations, neither at 13 DIV (Fig. 3B) nor at 26 DIV (Fig. 3C), suggesting that cultured cortical astrocytes do not express VGLUT2.

Second, we compared VGLUT2 expression in VGLUT2 WT, HZ, and KO mice using neuron/astrocyte cocultures (Fig. 4A), pure astrocytic cultures (Fig. 4B), and Western blots (Fig. 4C). Since VGLUT2 is expressed by thalamic relay neurons, neuron/astrocyte cocultures were prepared from the thalamus and maintained 15 DIV before fixation and triple-color immunostaining using antibodies against VGLUT2, GFAP to identify astrocytes (Nolte et al., 2001), and synapsin to visualize synaptic terminals (Fig. 4A). The VGLUT2 fluorescence intensity was normalized to synapsin or GFAP fluorescence to compare among images and samples. As expected, the VGLUT2/synapsin ratios were significantly different in cocultures from VGLUT2 WT/HZ and KO

mice, confirming the neuronal expression of the VGLUT2. In contrast, we found no significant difference in VGLUT2/GFAP ratios between neuron/astrocyte cocultures and between pure astrocytic cultures from VGLUT2 WT, HZ, and KO mice. Likewise, the densities of fluorescent puncta detected with VGLUT2 immunolabeling were similar in pure astrocytic cultures from VGLUT2 WT, HZ, and KO mice (Fig. 4B). Finally, the faint signal seen in the Western blots from cultured astrocytes was similar in the VGLUT2 WT, HZ, and KO preparations, whereas a strong specific band was present in the whole brain homogenate and a weak but clearly visible signal appeared in the cultured hippocampal neurons (DIV 18; Fig. 4C), which express very low levels of VGLUT2 (Wojcik et al., 2004). These results indicate a lack of specific VGLUT2 labeling in cultured astrocytes.

VGLUT3 expression in cultured astrocytes

Unlike VGLUT1 and VGLUT2, which are expressed by glutamatergic neurons, VGLUT3 is found in GABAergic, cholinergic, and monoaminergic neurons (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002). There is also some indication that astrocytes *in situ* express VGLUT3 (Fremeau et al., 2002), but again, this expression remains to be confirmed by appropriate controls with KO mice.

We first compared VGLUT3 immunostaining in hippocampal slices from VGLUT3 KO mice and WT littermates (Fig. 5A). After fixation, the slices were double labeled with antibodies against VGLUT3 and the plasma membrane type-1 glutamate transporter (GLT-1) (Chaudhry et al., 1995). VGLUT3-positive puncta seen in stratum pyramidale (SP), stratum radiatum (SR), and stratum oriens of the CA1 region in WT mice, which correspond probably to axon terminals of cholecystokinin-positive GABAergic interneurons (Somogyi et al., 2004), were not detected in the VGLUT3 KO mice, confirming the specificity of the VGLUT3 signal. Second, we compared the expression of VGLUT3 in cultured cortical astrocytes from VGLUT3 KO and WT littermates (Fig. 5B). Astrocytes were double-immunostained with antibodies against VGLUT3 and GFAP for normalization. The VGLUT3/GFAP ratio and the density of VGLUT3-positive puncta were similar in VGLUT3 KO and WT mice, indicating that the VGLUT3 signal in cultured astrocytes is unspecific. Third, Western blots of cultured cortical astrocytes from VGLUT3 WT, HZ, and KO littermates showed no specific VGLUT3 band at the correct molecular weight similar to the band seen in the striatum homogenate from VGLUT3 WT, which disappeared in KO striatal samples (Fig. 5C). The VGLUT3 protein produces a higher background signal than the other two VGLUT antibodies, but none of the additional bands disappear in the KO samples. Thus, taken together, our results indicate that also for VGLUT3, the weak signal seen in cultured astrocytes is due to nonspecific labeling.

VGLUT1 expression in brain slices

Since astrocytes in culture differ from their *in situ* counterparts (Cahoy et al., 2008), we further investigated the expression of all three VGLUT proteins in brain slices. We used double or triple immunostaining with specific antibodies against neuronal and astrocytic proteins, together with confocal microscopy, and quantitative colocalization methods in adult (P55–P65) WT and Tg mice. Confocal images were taken in the primary somatosensory (S1), hippocampal, and cerebellar cortex as well as in the thalamic ventrobasal (VB) and reticularis nuclei.

As expected (Fremeau et al., 2001; Herzog et al., 2001; Varoqui et al., 2002; Hioki et al., 2003; Boulland et al., 2004; Fremeau et al., 2004a; Nakamura et al., 2005; Graziano et al., 2008), VGLUT1

(Figure legend continued.) Examples of density-coded scatter plots for subregions of several pairs of labeling. The position of the data points depends on the normalized relative intensity of corresponding pixels from image pairs displayed in one dimension along the x- and y-axis. Dashed lines indicate the intensity of the background plus three times its SD; the top right quadrant contains the “red” and “green” pixels that are significantly different from background. Only the data points exceeding the dashed lines were used for calculating the Pearson’s correlation coefficient, r_{12} . The density of the data points is color encoded and shows the heterogeneous distribution of pixel–intensity ratios. **C**, The colocalization between VGLUT1 and several markers was quantified by the Pearson’s correlation coefficient r_{12} ($n = 3$ mice for synapsin; $n = 2$ for PSD-95; $n = 4$ for S100 β ; $n = 3$ for GLT-1; $n = 1$ for AQ4). The number of regions analyzed is given on the top of each bar. **D**, Pearson’s r_{12} values against the pixel offset shows an immediate decrease with pixel shift for VGLUT1/synapsin and VGLUT1/PSD-95, which contrasts with the slight increase seen for VGLUT1/S100 β , and a delayed and small shift for VGLUT1/GLT-1 and VGLUT1/AQ4 ($n = 7$).

immunostaining and low-magnification ($\times 10$) confocal imaging confirmed a high level of VGLUT1 expression in the neuropil of the neocortex, and in hippocampal and cerebellar regions of adult mice (data not shown). An earlier study using immunostaining and electron microscopy suggested that VGLUT1 is expressed by thin astrocytic processes in hippocampus (Bezzi et al., 2004). However, recent experiments using VGLUT1 KO mice indicate that a residual postembedding immunogold labeling was still present in hippocampal astrocytic processes ($\sim 60\%$) and synaptic terminals ($\sim 45\%$) (Ormel et al., 2012b), suggesting nonspecific labeling. Therefore, we studied the possible expression of VGLUT1 by astrocytes in S1 cortex (Fig. 7), as well as in hippocampal and cerebellar areas (see Fig. 8) using dual-color confocal imaging.

Comparing the distribution of the synaptic and astrocytic glutamatergic markers at the tripartite synapse is technically challenging because the astrocytes extend thin processes near the excitatory synapses (Ventura and Harris, 1999; Genoud et al., 2006), and the distance between synaptic and astrocytic compartments is close to or even below the spatial resolution of the confocal microscope (~ 150 nm with a $\times 63/\text{NA } 1.4$ objective at 550 nm). To compare the colocalization between VGLUT1 and astrocytic or neuronal markers, we used standardized fluorophore pairs (Table 2), calculated the Pearson's correlation coefficient (r_{12}), and examined the effect of an artificially introduced pixel shift between the two component images (see Materials and Methods).

To assess our ability to reliably detect colocalization, controls were performed for three astrocytic markers, S100 β , GLT-1, and the water-permeable channel aquaporin 4 (AQ4) (Fig. 6). Similar intensity line profiles and Pearson's correlation coefficient were obtained when comparing the distribution of the cytosolic marker, S100 β , and the membrane marker, GLT-1, in the neuropil of S1 cortex, CA1/CA3 hippocampal regions, and cerebellum (Fig. 6A–C). The correlation obtained for S100 β /GLT-1 was slightly smaller than that of VGLUT1/synapsin (+CTR) calculated in the same regions (Figs. 6C, 7), probably because of the plasma membrane (GLT-1) versus cytoplasmic (S100 β) distribution of these glial proteins. To validate this hypothesis, we performed a colocalization experiment between GLT-1, S100 β , and AQ4, another astrocyte-specific membrane marker mostly found at the glial endfeet but also present at the tripartite synapse (Nielsen et al., 1997; Binder et al., 2012). As expected, the correlation level between the membrane markers GLT-1 and AQ4 (Fig. 6D) was significantly higher than that of S100 β /GLT-1 ($p < 0.01$;

Fig. 6C) or S100 β /AQ4 ($p < 0.01$; Fig. 6E). The true colocalization between astrocytic markers was confirmed by a sharp decrease in Pearson's coefficient between S100 β /GLT-1 (50% over 473.4 ± 77.2 nm; Fig. 6C), AQ4/GLT-1 (50% over 593.1 ± 131.5 nm; Fig. 6D), and S100 β /AQ4 (50% over 532.7 ± 96.3 nm; Fig. 6E) induced by pixel shift. These controls validate our ability to demonstrate the colocalization of proteins inside submicrometric astrocytic processes using dual-color confocal images.

We then compared the distribution of VGLUT1 with that of two neuronal markers (synapsin, and PSD-95), and three astrocytic markers (S100 β , GLT-1, and AQ4) in the neuropil of S1 cortex (Fig. 7), hippocampal region (Fig. 8A–C), and cerebellum (Fig. 8D–F). We first analyzed the VGLUT1/synapsin and VGLUT1/PSD-95 line profiles, density-coded scatter plots, and Pearson's correlation coefficient and its evolution when introducing a pixel shift. Whereas VGLUT1 and synapsin line profiles showed substantial overlap as expected for two presynaptic proteins, VGLUT1 and PSD-95 had slightly shifted profiles, compatible with their expression in two adjacent but different compartments (Fig. 7A). For the same reasons, the density-coded scatter plots were symmetrical (along the 45° line of strong colocalization) for VGLUT1/synapsin, but asymmetrical for VGLUT1/PSD-95 (Fig. 7B). Finally, Pearson's correlation coefficient was significantly higher for VGLUT1/synapsin than for VGLUT1/PSD-95 ($r_{12} = 0.66 \pm 0.04$, $n = 19$ vs 0.24 ± 0.06 , $n = 12$, respectively; $p < 0.0001$; Fig. 7C), and, in both cases, r_{12} quickly dwindled when introducing a pixel shift as expected for synaptic proteins, which belong to subcellular compartments that are tightly associated within a diffraction-limited volume (Fig. 7D). This second set of control experiments indicates that our analysis can discriminate the VGLUT1 spatial correlation with a presynaptic marker, from its correlation with a postsynaptic marker.

We next compared the distribution of the astrocyte proteins, S100 β , GLT-1, and AQ4, against VGLUT1. For all three VGLUT1 versus astrocyte marker combinations, the line profiles indicated that the labeled astrocyte processes are close by but show little overlap with VGLUT1 (Fig. 7A). Second, the density-coded scatter plots showed less correlation between VGLUT1 and astrocytic markers than between VGLUT1 and synapsin or even PSD-95 (Fig. 7B). Third, the VGLUT1/S100 β correlation coefficient was close to zero and significantly lower than that of VGLUT1/PSD-95 ($r_{12} = -0.04 \pm 0.07$, $n = 22$ vs 0.24 ± 0.06 , $n = 12$; $p < 0.001$; Fig. 7C). Unlike the r_{12} values for VGLUT1/synapsin and VGLUT1/PSD-95 that both rapidly decreased upon pixel shift, the VGLUT1/S100 β coefficient instead showed a slight increase (Fig. 7D), indicating that S100 β -positive astrocytic compartments are juxtaposed to synapses, but not identical with the VGLUT1 expressing compartments. We also found that the VGLUT1/GLT-1 (0.23 ± 0.04 , $n = 18$) and VGLUT1/AQ4 (0.17 ± 0.05 , $n = 7$) r_{12} value failed to exceed that for VGLUT1/PSD-95 (0.24 ± 0.06 , $n = 12$; Fig. 7C), and the pixel shift did not produce a rapid falloff of the r_{12} value of VGLUT1/GLT-1 and VGLUT1/AQ4 as in the case of VGLUT1/synapsin or VGLUT1/PSD-95 (Fig. 7D). These results, like the VGLUT1/S100 β data, again suggest that GLT-1- and AQ4-positive astrocytic processes do not overlap with but are juxtaposed to VGLUT1-expressing cellular compartments. The different evolution of r_{12} with pixel shift points to different geometric arrangements with a partial and variable ensheathment of synapses by astroglial processes, and a stereotyped apposition of the presynaptic boutons and postsynaptic spines.

Using the same comparative analysis with line profiles and Pearson's correlation coefficient, quantitatively similar results

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(Figure legend continued.) contrasts with the overlapping line profiles of VGLUT1 and synapsin as shown here in the fascia dentate region. Scale bar, 20 μm . **B**, Colocalization quantification by Pearson's correlation coefficient r_{12} values between VGLUT1 and different markers in hippocampal regions. Three mice were used in each condition, except for VGLUT1/GLT-1 analysis in CA3 ($n = 2$ mice), VGLUT1/PSD-95 ($n = 1$ mouse), and VGLUT1/AQ4 ($n = 1$ mouse). There is no significant difference between VGLUT1/PSD-95 and VGLUT1/GLT-1 r_{12} values (n.s., $p > 0.16$). **C**, Effect of pixel shift on the r_{12} values between VGLUT1 and different markers. Data between VGLUT1 and synapsin, PSD-95, and AQ4 ($n = 10$) are pooled for hippocampus. **D**, High-magnification confocal images of the cerebellum and magnified ROIs ($14 \times 14 \mu\text{m}$). Scale bars: 20 μm . The fluorescence line profiles showed no correlation between VGLUT1 and the astrocytic markers S100 β , GLT-1, and AQ4. **E**, Pearson's r_{12} values in cerebellar ML and GL. Four mice were used for VGLUT1/S100 β analysis, three for VGLUT1/synapsin and VGLUT1/GLT-1, and one for VGLUT1/PSD-95. In cerebellum, the r_{12} values between VGLUT1 and astrocytic markers appear significantly smaller than the VGLUT1/PSD-95-negative control value. **F**, The effect of pixel shift on the r_{12} values confirms the lack of colocalization between VGLUT1 and the astrocytic markers, S100 β , GLT-1, and AQ4 ($n = 6$, pooled value from ML and GL regions).

Figure 9. Venus and astrocytic markers do not colocalize in the transgenic VGLUT1^{Venus} KI mice. **A**, Triple-color low-magnification confocal images of CA1, fascia dentate, and CA3 hippocampal regions from the Tg VGLUT1^{Venus} KI mouse immunostained with antibodies against S100 β , GLT-1, and VGLUT1. Scale bar, 100 μ m. **B**, Dual-color high-magnification confocal images of CA1 SR hippocampal (Hip) and cortical (Cx) regions from VGLUT1^{Venus} KI mice immunostained with antibodies against VGLUT1, synapsin, and PSD-95. Scale bar, 5 μ m. White arrows in the magnified ROI indicate the position of line profile. **C**, Dual-color high-magnification confocal images of cortical slices from VGLUT1^{Venus} KI mice immunostained with antibodies against S100 β (left) and GLT-1 (right). Scale bar, 20 μ m. **D–F**, Colocalization between Venus and various markers in brain slices quantified with Pearson's correlation coefficient (left) and offset-correlation (Figure legend continues.)

were obtained in the neuropil of the CA1, CA3, and dentate regions of the hippocampus (Fig. 8*A–C*) and in the molecular (ML) and granule cell (GL) layers of the cerebellum (Fig. 8*D–F*). In both hippocampal and cerebellar regions, the VGLUT1/synapsin line profiles overlapped (Fig. 8*A, D*, right) and showed high Pearson's correlation coefficient values ($r_{12} = 0.72 \pm 0.05$, $n = 23$ in hippocampus, Fig. 8*B*; $r_{12} = 0.71 \pm 0.05$, $n = 12$ in cerebellum, Fig. 8*E*), which were quickly reduced by pixel shift (Fig. 8*C, F*), as expected for overlapping correlated distributions of VGLUT1 and synapsin. The VGLUT1/PSD-95 line profiles were slightly shifted compared with VGLUT1/synapsin in both hippocampus and cerebellum (Fig. 8*A, D*, right), their correlation coefficients were smaller ($r_{12} = 0.19 \pm 0.09$, $n = 4$ in hippocampus, Fig. 8*B*; $r_{12} = 0.38 \pm 0.06$, $n = 6$ in cerebellum, Fig. 8*E*) and reduced by pixel shift (Fig. 8*C, F*). In neither region, the VGLUT1/S100 β line profiles were superimposed (Fig. 8*A, D*, left) and the correlation coefficient values were negative ($r_{12} = -0.07 \pm 0.08$, pooled value for hippocampus; $r_{12} = -0.09 \pm 0.1$ for cerebellum, respectively; Fig. 8*B, E*), significantly different from the VGLUT1/PSD-95 coefficient, and barely affected by pixel shift (Fig. 8*C, F*), indicating uncorrelated distributions of VGLUT1 and S100 β . In both regions, the VGLUT1/GLT-1 and VGLUT1/AQ4 line profiles were not superimposed (Fig. 8*A, D*, middle), and their weakly positive r_{12} values close to or lower than the VGLUT1/PSD-95 coefficient ($r_{12} = 0.16 \pm 0.1$, pooled value for hippocampus; $r_{12} = 0.15 \pm 0.08$ for cerebellum, respectively; Fig. 8*B, E*). The r_{12} value of VGLUT1/GLT-1 was not affected by pixel shift, as similarly observed for VGLUT1/AQ4 (Fig. 8*C, F*), indicating uncorrelated distributions between VGLUT1 and astrocytic membrane markers GLT-1 and AQ4. Together, these results using brain slices, immunostaining, and confocal microscopy do not provide evidence for the expression of VGLUT1 by S100 β , GLT-1, or AQ4-positive astrocytic processes of the cortex, hippocampus, and cerebellum.

Finally we investigated a possible colocalization of the astrocytic markers, GLT-1 and S100 β , with VGLUT1 using Tg VGLUT1^{Venus} KI mice (Herzog et al., 2011). The correlation between the distribution of Venus and the neuronal (VGLUT1, synapsin, PSD-95) or astrocytic (S100 β , GLT-1) markers was quantified in fixed cortical, hippocampal, and cerebellar slices (Fig. 9). We first examined the specificity of the Venus expression using antibodies against three neuronal proteins (VGLUT1, synapsin, and PSD-95). Low-magnification confocal images confirmed that Venus and VGLUT1 were similarly distributed in the hippocampus (Fig. 9*A*). The colocalization between Venus/VGLUT1 and Venus/synapsin is evident from their overlapping line profiles (Fig. 9*B*), the high positive correlation ($r_{12} = 0.72 \pm 0.04$, pooled value from all regions for Venus/VGLUT1; $r_{12} = 0.68 \pm 0.05$ for Venus/synapsin), and the swift decrease of r_{12} values upon pixel shift (Fig. 9*D–F*). Similar results were obtained when we quantified the Venus fluorescence itself (Fig. 9*D–F*, black labels) and when its fluorescence was enhanced by Venus immunostaining using an antibody against GFP (Fig. 9*D–F*, green labels). These control experiments confirm the specific targeting of Venus to VGLUT1-positive synapses (Herzog et al.,

2011). Our data show also that Venus/PSD-95 and VGLUT1/PSD-95 colocalization parameters are indistinguishable. However, by the same analysis, we found no evidence for colocalization between Venus and the two astrocyte markers, S100 β and GLT-1. In fact, in all brain regions studied (Fig. 9*D–F*), the Venus/S100 β correlation coefficient was negative and significantly lower than the Venus/PSD-95 coefficient ($r_{12} = 0.22 \pm 0.06$, $n = 10$, cortex; $r_{12} = 0.12 \pm 0.08$, $n = 12$, hippocampus; $r_{12} = 0.24 \pm 0.05$, $n = 7$, cerebellum), as was the Venus/GLT-1 coefficient ($r_{12} = 0.093 \pm 0.03$, $n = 10$, cortex; $r_{12} = 0.038 \pm 0.064$, $n = 17$, hippocampus; $r_{12} = 0.045 \pm 0.037$, $n = 14$, cerebellum). Again, these results using VGLUT1^{Venus} KI mice, immunostaining, and confocal microscopy do not provide any argument in favor of the expression VGLUT1 by the S100 β or GLT-1-positive astrocytic processes.

VGLUT2 expression in brain slices

The expression of VGLUT2 in the forebrain is sparse and mostly found at VGLUT1-negative synapses. Since VGLUT2 is found in the lemniscal axonal endings in the VB thalamic nucleus (Fremau et al., 2001; Herzog et al., 2001; Sakata-Haga et al., 2001), the thalamocortical axonal endings in the layer 4 S1 barrel cortex (Nakamura et al., 2005; Graziano et al., 2008), and the climbing fibers in the cerebellum (Hioki et al., 2003; Boulland et al., 2004; Mandolesi et al., 2009), we compared the distribution of VGLUT2, S100 β , and GLT-1 in the thalamic VB nucleus, layer 4 barrel cortex, and cerebellum, using neuronal and astrocytic markers and high-magnification confocal immunofluorescence imaging (Fig. 10).

Using the same type of pixel-based colocalization analysis, we found no evidence for VGLUT2/synapsin colocalization in these regions (Fig. 10*D–F*). Thus, VGLUT2- and VGLUT1-positive synapses that express different SNARE proteins (Mandolesi et al., 2009), probably express also different regulatory synaptic proteins, such as synapsin, which was detected only at VGLUT1-positive synapses. As a consequence we could not use the VGLUT2/synapsin Pearson's values as a positive control. Instead, we used the VGLUT1/synapsin correlation values found in both cortex (Fig. 7) and hippocampus (Fig. 8*A, B*). In the thalamic VB nucleus where VGLUT1 is expressed by the synapses of cortical origin (Graziano et al., 2008), we found overlapping VGLUT1 and synapsin fluorescence profiles (Fig. 10*B*), a correlated scatter plot (Fig. 10*C*), and a high correlation that rapidly dwindled when introducing a pixel shift between the VGLUT1 and synapsin images ($r_{12} = 0.74 \pm 0.01$, $n = 5$, Fig. 10*D*). Finally, as in the cortex (Fig. 7) and hippocampus (Fig. 8*A, B*), in the VB nucleus, we found nonoverlapping VGLUT1 and PSD-95 fluorescence profiles (Fig. 10*B*), a weakly correlated scatter plot (Fig. 10*C*), and a Pearson's value that was relatively weak and immediately reduced by pixel shift ($r_{12} = 0.19 \pm 0.04$, $n = 5$, Fig. 10*D*), as expected for proteins expressed by two nearby distinct compartments. Therefore we used VGLUT1/synapsin as a positive control (+CTR), and VGLUT1/PSD-95 as a negative control (–CTR) for quantifying protein distributions (Fig. 10*D–F*). In the same brain regions, VGLUT2/S100 β and VGLUT2/GLT-1 fluorescence profiles (Fig. 10*A*), scatter plots (Fig. 10*C*), and correlation analysis (Fig. 10*D–F*) did not lend any support to a colocalization between VGLUT2 and the astrocyte markers, S100 β and GLT-1. Their correlation was indistinguishable from the negative control. Our results indicate that neither the astrocytes in the thalamic VB nucleus, nor in the layer 4 barrel cortex, nor in the cerebellum express VGLUT2.

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(Figure legend continued.) plot (right). Along with directly imaging Venus fluorescence, we also used an antibody against GFP to increase the Venus signal. Similar results were obtained with (green outline) and without the GFP antibody, confirming that the Venus fluorescence itself is strong enough for quantitative imaging. Similar experiment and analysis were performed for cortical (*D*), hippocampal (*E*), and cerebellar (*F*) regions. Two VGLUT1^{Venus} KI mice were used.

Figure 10. VGLUT2 and astrocytic markers do not colocalize. **A**, Dual-color high-magnification confocal images in the VB thalamic nucleus immunostained with antibodies against VGLUT2, synapsin, PSD-95, S100β, and GLT-1. Scale bars, 20 μm. **B**, Magnified ROI in the VB thalamic nucleus immunostained with antibodies against VGLUT1 and synapsin for positive (Figure legend continues.)

VGLUT3 expression in brain slices

Unlike VGLUT1 and VGLUT2, VGLUT3 is expressed by cells that are not recognized as conventional glutamatergic neurons such as the raphe serotonergic neurons, the striatal cholinergic neurons, and subtypes of hippocampal and cortical GABAergic interneurons (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002; Somogyi et al., 2004). VGLUT3 is also expressed by non-neuronal cells such as inner hair cells of the ear (Ruel et al., 2008; Seal et al., 2008), where its mutation or its genetic inactivation leads to deafness. Interestingly, VGLUT3 inactivation leads to cortical hyperexcitability and to a deficit of neuronal growth (Seal et al., 2008). Finally, a putative astrocytic expression of VGLUT3 (Fremeau et al., 2002; Ormel et al., 2012a) is still controversial since Western blot analysis was unable to demonstrate the expression of VGLUT3 in the cerebellum (Gras et al., 2002).

To investigate the possible expression of VGLUT3 by astrocytes *in situ*, we compared the distributions of VGLUT3, S100 β , and GLT-1 in hippocampal and cortical slices (Fig. 11). We used the mean VGLUT1/synapsin and VGLUT1/PSD-95 correlation coefficients as positive (+CTR) and negative (–CTR) controls, respectively (Fig. 11C,D). Neither VGLUT3/GLT-1 (Fig. 11A) nor VGLUT3/S100 β intensity profiles showed overlap (Fig. 11B). The VGLUT3/S100 β correlation was close to zero in hippocampal ($r_{12} = 0.005 \pm 0.02$, $n = 13$) and cortical ($r_{12} = 0.03 \pm 0.04$, $n = 6$) regions, being significantly lower than the VGLUT1/PSD-95 negative controls ($r_{12} = 0.19 \pm 0.09$, $n = 4$ in hippocampus; $r_{12} = 0.25 \pm 0.06$, $n = 12$, in cortex; $p < 0.001$; Fig. 11C,D). The VGLUT3/S100 β coefficient was also unchanged upon pixel shift, indicating a lack of correlation between VGLUT3 and S100 β . Likewise, the weak correlation between cortical VGLUT3 and GLT-1 signals was significantly smaller than the VGLUT1/PSD-95 control. In the hippocampus, no difference was found between VGLUT3/GLT-1 and VGLUT1/PSD-95 correlation coefficients. Together, these results do not provide any evidence for the colocalization VGLUT3 with S100 β or GLT-1, suggesting that neither cortical nor hippocampal astrocytes express VGLUT3 *in situ*.

Discussion

Our data provide no evidence in favor of the expression of any of the three vesicular glutamate transporters (VGLUT1–3) by the gray matter protoplasmic astrocytes found in the primary somatosensory cortex and the thalamic VB nucleus, as well as the hippocampus and the cerebellum. We first used VGLUT1–3 KO mice, VGLUT1^{Venus} KI, immunostaining, and Western blots, and we could not detect the expression of VGLUT1–3 in cortical and thalamic astrocytes in culture (Figs. 1–5). Second, using immunostaining and confocal imaging of brain slices, we quantified the degree of overlap between VGLUT1–3 and neuronal or astrocytic markers with fluorescence line profiles, dual-color scatter plots, and Pearson's correlation coefficient analysis, again finding

no evidence for colocalization between VGLUT1–3 and astrocytic markers (Figs. 6–11).

Astrocytes in culture do not express VGLUT1–3

Our experiments confirmed earlier observation that immunostaining against VGLUT1 (Montana et al., 2004; Zhang et al., 2004; Anlauf and Derouiche, 2005; Bowser and Khakh, 2007; Stenovec et al., 2007; Marchaland et al., 2008) and VGLUT2 (Montana et al., 2004; Anlauf and Derouiche, 2005; Crippa et al., 2006; Ni and Parpura, 2009) labels astrocytes in culture (Figs. 1, 4). Similar immunostaining was observed with VGLUT3 antibodies (Fig. 5). However, for all three VGLUTs the immunostaining was relatively weak and unaffected by the genetic ablation of the VGLUTs, indicating that the astrocytes in culture lack VGLUT1–3, and that the faint punctate labeling was not specific. The lack of VGLUT1 expression by the astrocytes in culture was also confirmed using live-cell TIRFM showing the absence of Venus-positive vesicles in the astrocytes in culture prepared from VGLUT1^{Venus} KI mice (Fig. 2). Finally, no evidence for VGLUT2 expression could be obtained in the cultured astrocytes from the Tg Bac VGLUT2-EGFP mice (Fig. 3).

VGLUT1–3 immunofluorescence does not correlate with astrocytic markers in cortical slices

We used the Pearson's correlation coefficient to quantify the spatial correlation between VGLUT1–3, and various neuronal or astrocytic markers *in situ* using confocal images of fixed brain slices. First, our control experiments with antibodies against three astrocytic proteins (S100 β , a cytosolic Ca²⁺ binding protein; GLT-1, a transmembrane glutamate transporter; AQ4, a transmembrane water-permeable channel), which label the thin astrocytic processes, demonstrated overlapping fluorescence profiles and high correlation that rapidly dropped with pixel shift in all regions investigated, as expected for proteins expressed by the thin subdiffraction astrocytic processes (Fig. 6). Second, to estimate meaningful upper (+CTR) and lower (–CTR) boundaries of our correlation estimates, we performed dual-color immunostaining with antibodies against VGLUT1 and the presynaptic marker synapsin (+CTR), or the postsynaptic density marker PSD-95 (–CTR). Our results show that our method is able to distinguish between true colocalization of VGLUT1 and synapsin, and diffraction-limited partial overlap of VGLUT1 and PSD-95 fluorescence signal. The VGLUT1/synapsin fluorescence profiles showed complete overlap, with linear dual-color scatter plots, and maximal Pearson's values that were quickly reduced by pixel shift (Figs. 7–10). The VGLUT1/PSD-95 fluorescence showed nonoverlapping profiles with distorted dual-color scatter plots, and smaller correlation coefficients that were nonetheless reduced by pixel shift (Figs. 7–10). These results show that our quantification analysis can distinguish truly overlapping compartments and nonoverlapping diffraction-limited neuronal and astroglial compartments found at the tripartite synapse (Ventura and Harris, 1999; Genoud et al., 2006).

A recurrent result of our study is the lack of overlap between the three VGLUTs and three astrocytic markers, S100 β , GLT-1, and AQ4. Pearson's correlation coefficients between all three VGLUT1–3 proteins and the astrocytic cytosolic marker S100 β were close to zero and significantly smaller than the negative controls (–CTR) of our colocalization analysis (Figs. 7–10), indicating that the VGLUT1–3 proteins do not locate within the astrocytic cytosol in all regions we examined. This conclusion has been further confirmed by the absence of a steep reduction of the VGLUT/S100 β Pearson's correlation coefficient upon pixel shift,

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(Figure legend continued.) control (+CTR), and against VGLUT1 and PSD-95 for negative control (–CTR). Scale bar, 5 μ m. C, Density-coded scatter plots illustrating the correlation between pixel intensities of different pairs of markers. D–F, Pearson's correlation coefficient analysis and the offset-correlation plot quantifying the colocalization between VGLUT2 and astrocytic markers in thalamic (D), cortical (E), and cerebellar (F) slices. Unlike VGLUT1, VGLUT2 shows low colocalization with synapsin. In cortical and cerebellar regions, the mean value of +CTR and –CTR is taken from VGLUT1 experiments (Figs. 7, 8), and the error bars omitted for clarity. Two animals were used for each VGLUT2 experiment, and one animal used for VGLUT1-synapsin/PSD-95 experiment in thalamus.

Figure 11. VGLUT3 and astrocytic markers do not colocalize. Dual-color high-magnification confocal images in hippocampal slices coimmunostained with antibodies against VGLUT3 and GLT-1 in the CA3 region (**A**), and against VGLUT3 and S100 β in CA1 region (**B**). Scale bars: 20 μ m. **C, D**, Quantitative colocalization analysis using Pearson's correlation coefficient and the offset-correlation plot in hippocampus (**C**) and cortex (**D**). The mean values of VGLUT1/synapsin (+CTR) and VGLUT1/PSD-95 (–CTR) were taken from VGLUT1 experiments (Figs. 7, 8). Number of analyzed regions in one mouse is indicated above each bar.

which rather shows no change or, if any, a slight increase, implying proximity or a loose apposition, rather than colocalization, between the VGLUT proteins and S100 β -positive astrocytic processes, as expected from electron micrographs (Ventura and Harris, 1999; Genoud et al., 2006). Our results comparing the distribution of VGLUTs and the astrocytic transmembrane protein, GLT-1 and AQ4, were slightly different, but they also failed to show overlap between VGLUT proteins and GLT-1/AQ4-positive astrocytic processes. The correlation coefficients with VGLUT labeling were larger for membrane proteins GLT-1 and AQ4 than for the cytoplasmic Ca²⁺-binding protein S100 β , but they failed to exceed the level of the VGLUT1/PSD-95-negative control, and their offset-correlation plots were unchanged with pixel shift (Figs. 7, 8B, 9, 10). Thus, the low correlation coefficient between VGLUT and astrocytic membrane markers GLT-1 and AQ4 is likely to reflect the diffraction limit-induced overlap of the fluorescence of the two types of proteins that reside in two distinct compartments separated by a distance below the spatial resolution of confocal microscopy, as expected by the proximity between astrocytic membrane and the presynaptic compartment (Ventura and Harris, 1999; Genoud et al., 2006).

In conclusion, our results from brain slices provide no evidence that VGLUT proteins are present in astrocytes *in situ*. Thus the global pixel-based analysis corroborates our single-vesicle data from cultured astrocytes. Together, our results therefore call into reconsideration the molecular mechanism by which astrocytes participate in the glutamate-mediated astrocyte-to-neuron signal at tripartite synapse. Ca²⁺-regulated lysosomal exocytosis identified in astrocytes (Zhang et al., 2007; Li et al., 2008) is one possible candidate pathway for glutamate exocytosis since the lysosomal sialic acid transporter, sialin, has been reported to behave as an aspartate/glutamate transporter that accumulates the amino acids inside the vesicular compartment (Miyaji et al., 2008). However lysosomal exocytosis operates on a timescale much slower than the Ca²⁺-dependent exocytosis recorded in astrocytes (Bezzi et al., 2004; Zhang et al., 2007; Li et al., 2008; Marchaland et al., 2008). Other routes for glutamate release from astrocytes have been suggested, such as hemichannels (Ye et al., 2003), anion channels (Takano et al., 2005; Kimelberg et al., 2006; Park et al., 2009; Li et al., 2012), and reversed glutamate transport (Rossi et al., 2000). Their possible participation in various physiological and pathological conditions will require more studies (Cali et al., 2009; Fiacco et al., 2009; Perea et al., 2009; Halassa and Haydon, 2010; Hamilton and Attwell, 2010).

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