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GABAergic basket cells expressing cholecystokinin contain vesicular glutamate transporter type 3 (VGLUT3) in their synaptic terminals in hippocampus and isocortex of the rat

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Abstract

Vesicular glutamate transporter type 3 (VGLUT3) containing neuronal elements were characterized using antibodies to VGLUT3 and molecular cell markers. All VGLUT3-positive somata were immunoreactive for CCK, and very rarely, also for calbindin; none was positive for parvalbumin, calretinin, VIP or somatostatin. In the CA1 area, $26.8 \pm 0.7\%$ of CCK-positive interneuron somata were VGLUT3-positive, a nonoverlapping $22.8 \pm 1.9\%$ were calbindin-positive, $10.7 \pm 2.5\%$ VIP-positive and the rest were only CCK-positive. The patterns of coexpression were similar in the CA3 area, the dentate gyrus and the isocortex. Immunoreactivity for VGLUT3 was undetectable in pyramidal and dentate granule cells. Boutons colabelled for VGLUT3, CCK and GAD were most abundant in the cellular layers of the hippocampus and in layers II–III of the isocortex. Large VGLUT3-labelled boutons at the border of strata radiatum and lacunosum-moleculare in the CA1 area were negative for GAD, but were labelled for vesicular monoamine transporter type 2, plasmalemmal serotonin transporter or serotonin. No colocalization was found in terminals between VGLUT3 and parvalbumin, vesicular acetylcholine transporter and group III (mGluR7a,b; mGluR8a,b) metabotropic glutamate receptors. In stratum radiatum and the isocortex, VGLUT3-positive but GAD-negative boutons heavily innervated the soma and proximal dendrites of some VGLUT3- or calbindin-positive interneurons. The results suggest that boutons coexpressing VGLUT3, CCK and GAD originate from CCK-positive basket cells, which are VIP-immunonegative. Other VGLUT3-positive boutons immunopositive for serotonergic markers but negative for GAD probably originate from the median raphe nucleus and innervate select interneurons. The presumed amino acid substrate of VGLUT3 may act on presynaptic kainate or group II metabotropic glutamate receptors.

Introduction

The cell bodies and proximal dendrites of cortical pyramidal cells are innervated exclusively by GABAergic terminals, as revealed by immunocytochemical labelling for glutamic acid decarboxylase (GAD; Ribak, 1978) and γ-amino butyric acid (GABA; Storm-Mathisen *et al*., 1983; Somogyi & Hodgson, 1985). At least two distinct populations of basket cells provide this somatic and perisomatic inhibition. One contains parvalbumin (PV; Katsumaru *et al*., 1988; Sik *et al*., 1995); the other expresses peptides derived from procholecystokinin (CCK; Nunzi *et al*., 1985; Freund *et al*., 1986). Some of these latter cells also express vasoactive intestinal polypeptide (VIP; Acsady *et al*., 1996). Although the relative weight of these two distinct GABAergic inputs may differ amongst various cortical principal cells, this dual GABAergic innervation is found throughout the whole cortical mantle.

Recent cloning (Fremeau, *et al*., 2002; Gras *et al*., 2002; Schafer *et al*., 2002; Takamori *et al*., 2002) and immunohistochemical localization of the subtype 3 vesicular glutamate transporter (VGLUT3) revealed that many terminals on cell bodies of hippocampal pyramidal cells and interneurons are immunoreactive for VGLUT3 (Fremeau *et al*., 2002; Gras *et al*., 2002), where VGLUT3-positive terminals establish symmetrical synapses (type II; Gray, 1959) similar to those made by GABAergic cells. This implies that at least one type of basket cell contains VGLUT3. Indeed, some GAD immunoreactive cells were labelled for VGLUT3 in the hippocampus (Fremeau *et al*., 2002). Therefore, we tested basket cell molecular markers for possible colocalization with VGLUT3.

Furthermore, the whole cortical mantle is innervated by cholinergic and serotonergic afferents and both cholinergic and serotonergic neurons were shown to express VGLUT3 (Fremeau *et al*., 2002; Gras *et al*., 2002; Schafer *et al*., 2002). Hence, we tested the contribution of

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extrinsic VGLUT3-positive afferents to the hippocampus by colabelling sections for VGLUT3 with vesicular transporters for acetylcholine and amines.

The functional role and natural substrate of VGLUT3 in GABAergic terminals are not known. Glutamate is transported into synaptic vesicles by VGLUT3 (Fremeau *et al*., 2002; Gras *et al*., 2002; Schafer *et al*., 2002; Takamori *et al*., 2002). However, in GABAergic terminals GAD, an enzyme that is anchored to synaptic vesicles (Jin *et al*., 2003), converts glutamate into GABA. Therefore, glutamate concentration in GABAergic terminals is likely to be low. Whatever molecule is transported by VGLUT3, it can be released with GABA and it may act on pre and/or postsynaptic excitatory amino acid receptors. Postsynaptic glutamate receptors have not been found in type II synapses established by GABAergic boutons on the soma of pyramidal cells; only the metabotropic glutamate receptor mGluR5 has been reported to be present at nonsynaptic sites at a low density (Lujan *et al*., 1996). However, GABA release is modulated by presynaptic kainate receptors (Kullmann, 2001) and both group II (Poncer *et al*., 2000) and group III mGluRs (Semyanov & Kullmann, 2000). These latter presynaptic receptors are of particular interest because they are enriched in the presynaptic active zone of both glutamatergic (Shigemoto *et al*., 1996; Shigemoto *et al*., 1997) and GABAergic cortical terminals (Dalezios *et al*., 2002; Somogyi *et al*., 2003), a position exposing them to a high concentration of the released transmitters. In order to test coexpression of VGLUT3 with presynaptic group III metabotropic receptors in terminals we carried out coimmunolabelling experiments in the hippocampus of the rat.

Materials and methods

Tissue preparation

Twelve male Wistar rats (150–250 g) were deeply anaesthetized with Sagatal (pentobarbitone sodium, 60 mg/mL, i.p.). Treatment of animals was carried out in accordance with the UK Animals (Scientific Procedure) Act of 1986 and associated procedures. All efforts were made to minimize the number of animals used. This project was approved by the Ethical Review Committee of Oxford University. The rats were perfused transcardially with 0.9% saline for approximately 1 min. This was followed by one of the two fixatives; a solution containing 4% paraformaldehyde, 0.05% glutaraldehyde and 15% (v/v) saturated picric acid made up in 0.1 M phosphate buffer (PB; pH 7.4), or the same solution but without glutaraldehyde. After perfusion, the brains were removed and stored in PB overnight at 4 °C. The forebrain was cut into 50–60 μm thick coronal sections with a vibratome and the sections were stored in PB.

Antibodies

Primary antibodies, which were reported previously, are listed and referenced in Table 1. New antibodies were raised to rat metabotropic glutamate receptor type 8, splice variant b (mGluR8b; Corti *et al*., 1998) in a rabbit (amino acid residues, SKSSVDFQMVKSGSTS; GenBank accession number, Y11153), to rat VGLUT3 in a guinea pig (522–588; NM153725), to rat vesicular monoamine transporter type 2 (VMAT2) in a rabbit (468–515; L00603) and to mouse plasmalemmal serotonin transporter (5-HTT) in a rabbit (1–77; AF013604). The production and purification of bacterially expressed glutathione-*S* transferase fusion proteins and affinity purification of immunoglobulins have been previously reported (Sakai *et al*., 2003). For immunoblots, the telencephalon of an adult Wistar rat was homogenized in eight to ten volumes of ice-cold buffer containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl (pH 7.10), and 0.4 mM phenylmethylsulphonyl fluoride using a Potter homogeniser with 15 strokes at 800 r.p.m. To remove nuclei and large debris the homogenate was centrifuged at $1000 \times \boldsymbol{g}$, for 10 min. The supernatant was centrifuged again at $10\,000 \times \boldsymbol{g}$, for 20 min. The resulting supernatant was hypoosmotically lysed and the lysate was centrifuged at $25\,000 \times \boldsymbol{g}$, for 30 min in order to obtain a fraction enriched in synaptosomal membranes. The supernatant was re-centrifuged at $165\,000 \times \boldsymbol{g}$ for 12 h, yielding the synaptic vesicle-enriched fraction in the pellet used for testing the antibodies to VGLUT3 (Fig. 1). The synaptosomal membrane fraction was re-suspended in the homogenising solution, complemented with 0.5% Triton X-100, for 15 min, and re-centrifuged at $111\,000 \times \boldsymbol{g}$ for 1 h, pelleting the membrane fraction containing presynaptic membranes, and this fraction was used to test the antibodies to 5-HTT and mGluR8b. Protein concentrations were determined by the method of Lowry *et al*. (1951). Following SDS-PAGE electrophoresis, the fractionated proteins were electroblotted onto nitrocellulose membranes (BioTraceNT, PALL, Ann Arbor, MI, USA). The membranes were incubated with 5% skimmed milk in Tris buffered saline (TBS, pH 7.5) containing 0.1% Tween 20 for 1 h, followed by incubation with affinity-purified primary antibodies (1 μg/mL) for 2 h. Immunoreaction was visualized with ECL chemiluminescence kit (Amersham, Bucks, UK).

Immunocytochemistry

Floating sections were incubated in 20% normal goat or donkey serum diluted in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl and 0.3% Triton X-100 (TBSTx) for 1 h followed by one or a mixture of two to three primary antibodies overnight at 4 °C. For combinations and dilutions, see Table 1. Then, TBSTx containing 1% normal serum and one or a mixture of second antibodies tagged with fluorophore were applied at 4 °C overnight. Between incubations, sections were washed in copious TBSTx. Finally, they were rinsed in PB and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

The newly raised antibodies to VGLUT3, VMAT2 and 5-HTT (1 μg/mL) were absorbed to the whole fusion protein (30 μg/mL), which eliminated all labelling. The absorption to glutathione-*S* transferase (30 μg/mL) alone did not change the immunolabelling of tissue sections. To test cross-reactivity between various antibodies in double and triple immunolabelling experiments, sections were processed with a full complement of secondary antibodies and one primary antibody at a time. None of the combinations with species-unrelated secondary antibodies resulted in labelling. No selective labelling was found in sections incubated with combinations of secondary antibodies without a primary antibody.

Imaging and quantification

Expression of VGLUT3 and CCK with either calbindin D28K (CB), calretinin (CR) or VIP was quantified in triple immunolabelled sections from the CA1 region of the hippocampus. The 3-D cell counting technique was implemented on a Zeiss LSM 510/Axiovert 100 M confocal microscope. Using 40× oil lens at 0.7× digital zoom, 325×325 μm areas of a 50 μm thick vibratome section were optically sectioned into 25 approximately 1 μm thick slices, spaced at 2 μm centre-to-centre intervals. In this way, a stack of 25 optical slices represented a $325 \times 325 \times 50$ μm block of tissue. Depending on the orientation of the section, a grid of 25–30 stacks covered an entire dorsal hippocampal CA1 region. Neighbouring stacks had an overlap of 20–25 μm. Each of the 25 optical sections in a stack was scanned and recorded for three fluorophores. Channel settings were for Alexa488, Argon laser (488 nm, Lasos LGK 7812 ML-1/LGN 7812, 25 mW), emission filter LP505; for Cy3, HeNe laser 1 (543 nm, Lasos LGK 7786 P, 1 mW), emission filter LP560; for Cy5, HeNe laser 2 (633 nm Lasos LGK 7628–1, 5 mW), emission filter LP650. The main

TABLE 1. Summary of antibodies, their sources, dilutions and combinations

Antibody	Species	Dilution	Combinations of antibodies in single, double and triple labelling experiments																													Secondary antibody	Source of primary antibody	References for characterization
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31			
GAD	Sheep	1 : 1000	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	Donkey antisheep Alexa 488 (Mol.Probes)	E. Mugnaini, University of Connecticut, USA	Oertel *et al.* (1980)
mGluR8a	Guinea pig	1 µg/mL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	Donkey antiguinea pig Alexa 488 (Mol. Probes)	R. Shigemoto, Natl. Institute Physiol., Okazaki, Japan	Kinoshita *et al.* (1996)
mGluR8b	Rabbit	1 µg/mL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	Donkey antirabbit Cy3 (Jackson)	R. Shigemoto, Natl. Institute Physiol., Okazaki, Japan	See Materials and methods
mGluR7a	Rabbit	1 µg/mL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	Donkey antirabbit Cy3 (Jackson)	R. Shigemoto, Natl. Institute Physiol., Okazaki, Japan	Shigemoto *et al.* (1996), 1997)
mGluR7b	Guinea pig	1 µg/mL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Donkey antiguinea pig Alexa 488 (Mol.Probes)	R. Shigemoto, Natl. Institute Physiol., Okazaki, Japan	Kinoshita *et al.* (1998)
mGluR1a	Rabbit	1 µg/mL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antirabbit Cy3 (Jackson)	DiaSorin, Stillwater, USA	Alvarez *et al.* (2000)
			–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antirabbit Cy5 (Jackson)		
PV	Guinea Pig	1 : 1000	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antiguinea pig Alexa 488 (Mol.Probes)	K.G. Baimbridge, University of British Columbia, Canada	Personal communication
			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	Donkey antiguinea pig Cy5 (Jackson)		
VIP	Mouse	1 : 5000	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	Donkey antimouse Cy3 (Jackson)	Biogenesis, Poole, England	Dey *et al.* (1988)
			–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antimouse Alexa 488 (Mol.Probes)		
VIP	Rabbit	1 : 200	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antirabbit Cy3 (Jackson)	Euro-Diagnostica, Malmö, Sweden	Ekblad *et al.* (1984)
SS	Mouse	1 : 500	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antimouse Cy5 (Jackson)	A. Buchan, MRC Reg. Peptide group, Canada	Vincent *et al.* (1985)
			–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antimouse Cy3 (Jackson)		
CR	Mouse	1 : 2000	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	Donkey antimouse Cy5 (Jackson)	Swant, Bellinzona, Switzerland	Schwaller *et al.* (1995)
CR	Rabbit	1 : 800	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antirabbit Cy5 (Jackson)	Biogenesis, Poole, England	Jacobowitz & Winsky (1991)
			–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	Donkey antirabbit Cy3 (Jackson)		
CB	Mouse	1 : 400	–	–	–	+	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	+	–	–	–	–	–	+	+	+	–	–	Donkey antimouse Cy5 (Jackson)	Swant, Bellinzona, Switzerland	Celio *et al.* (1990)
			–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antimouse Cy3 (Jackson)		
CB	Rabbit	1 : 500	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	Donkey antirabbit Cy5 (Jackson)	Swant, Bellinzona, Switzerland	
			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	Donkey antirabbit Cy3 (Jackson)		
VGLUT3	Rabbit	0.06 µg/mL	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antirabbit Cy5 (Jackson)	S. El Mestikawy, INSERM, France	Gras *et al.* (2002); P45-3 #2
			–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antirabbit Alexa 488 (Mol. Probes)		
			–	–	+	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	+	–	Donkey antirabbit Cy3 (Jackson)		
VGLUT3	Guinea pig	1 µg/mL	–	–	–	–	+	+	+	–	+	+	–	+	–	+	+	+	+	–	+	+	+	+	–	+	–	–	+	+	–	–	+	Donkey antiguinea pig Alexa 488 (Mol.Probes)	M. Watanabe, Hokkaido University, Japan	See Materials and methods
			–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antiguinea pig Cy5 (Jackson)		
			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	Donkey antiguinea pig FITC (Jackson)		
			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Donkey antiguinea pig Cy3 (Jackson)		
CCK	Rabbit	1 : 2000	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	Donkey antirabbit Cy5 (Jackson)	pro-CCK; A. Varro, Liverpool University	Morino *et al.* (1994)
			–	–	–	–	–	–	–	+	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Donkey antirabbit Cy3 (Jackson)	CCK-8; DiaSorin, Stillwater, USA	
VAChT	Goat	1 µg/mL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	Donkey antigoat Alexa 488 (Mol. Probes)	Chemicon, Temecula, USA	Arvidsson *et al.* (1997)
5-HTT	Rabbit	1 µg/mL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	Donkey antirabbit Cy3 (Jackson)	M. Watanabe, Hokkaido University, Japan	See Materials and methods
VMAT2	Rabbit	1 µg/mL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	Donkey antirabbit Cy3 (Jackson)	M. Watanabe, Hokkaido University, Japan	See Materials and methods
5-HT	Rabbit	1 : 2000	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	Donkey antirabbit Cy3 (Jackson)	Zymed Laboratories, Inc. San Francisco, USA.	
TH	Mouse	1 : 1000	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	Donkey antimouse Cy3 (Jackson)	Sigma	

Each column of + characters represents the mixture of antibodies for a reaction.

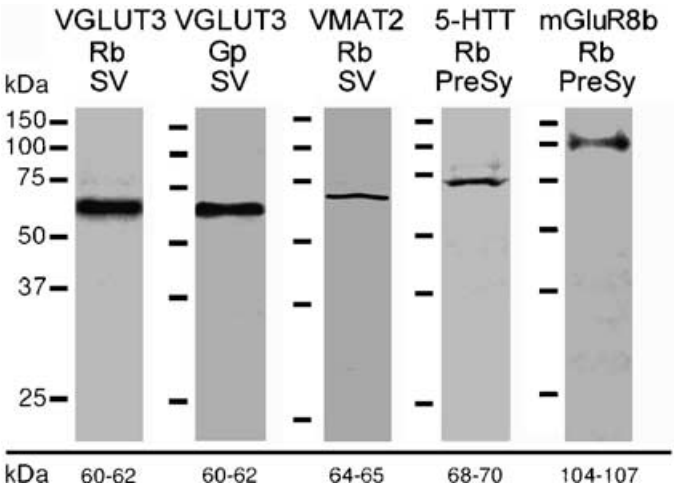


FIG. 1. Immunoblots of synaptic vesicle-enriched (SV) and presynaptic membrane fraction (PreSy) from rat forebrain. Expected molecular weights are indicated under each column. Rb, rabbit; Gp, guinea pig.

and secondary beam splitters (UV/488/543/633, NFT635 and NFT545) were the same. Pinhole sizes were chosen to keep the slices 1 μm thick. Each channel was set up in a different scanning track. Tracks were switched line by line in sequence of decreasing excitation wavelengths. During a line scan, only one excitation laser and the corresponding emission detector were active allowing the use of transparent, long-path emission filters. This arrangement significantly improved the sensitivity of detection. Output signals from two subsequent line scans were averaged. In each experiment, for each track, channel separation was tested by systematic cross-excitation and detection between the channels.

Boundaries of the CA1 region were determined, and labelled cells were located in the normal transparent projection of all the 25 slices. Perikarya were scrutinized for the three markers in a series of adjacent optical sections. Each triplet of markers was tested for somatic colocalization in three animals using one hemisphere per animal.

A semiquantitative assessment of VGLUT3-positive cells coexpressing either CR, CB or CCK was carried out in the CA3 area, dentate gyrus and hilar region of the dorsal hippocampus, as well as in the parietal isocortex using a conventional fluorescence microscope. A similar but separate survey was carried out to estimate VGLUT3 colabelling for PV, somatostatin (SS) or the metabotropic glutamate receptor type 1, splice variant α (mGluR1α) in the same areas, as well as in the CA1 region. In both estimations VGLUT3 immunoreactive cells were scored and tested for another marker as they were encountered under a 40× objective lens. In order to check the consistency of VGLUT3 expression in CCK-positive cells a similar tally was performed, but in this case CCK cells were identified first and then checked for VGLUT3 labelling. Each pair of markers was tested for colocalization in three animals, using one or two hemispheres per animal. For semiquantitative estimations, a Leitz DM RB epifluorescence microscope was used with the filter sets described earlier (Ferraguti *et al*., 2003). Images were recorded with CCD camera (Hamamatsu C4742–95). The stored digital images were visualized and analysed with Improvision software (Openlab, version 3.0.2).

In order to achieve the required high detection sensitivity for colocalization of cellular markers in boutons, a 63× oil lens was used. Though a 100× oil lens permits thinner optical slices, it provides a lower intensity image. To exploit the brightness of the 63× oil lens, pinhole sizes were kept in the range of one Airy unit. In this way, slightly less than 0.4 μm thick optical slices were produced.

In all imaging processes the thickness of the corresponding optical slices was the same, brightness and contrast were adjusted for a whole frame and no part of an image was modified separately in any way. In some of the illustrations, contrast and brightness were enhanced by overlaying adjacent optical slices. The images of 5-HT (serotonin) and VGLUT3 colabelling were taken with an Olympus Fluoview confocal microscope. The results are illustrated as black and white images due to the cost of colour illustrations.

Results

Antibodies

Antibodies to VMAT2, 5-HTT and mGluR8b recognized proteins with the expected molecular weight in immunoblots (Fig. 1), and in case of the first two antibodies labelled the brain in a pattern similar to that published for other antibodies to the same molecules (Peter *et al*., 1995; Zhou *et al*., 1996; Lebrand *et al*., 1998). The antibody to VMAT2 also slightly labelled the cell nuclei of all cells in a nonspecific manner. No difference was seen in the immunohistochemical labelling patterns and density between the newly raised antibody to VGLUT3 and that previously published by Gras *et al*. (2002). Therefore, they are not distinguished in the ensuing description of the results.

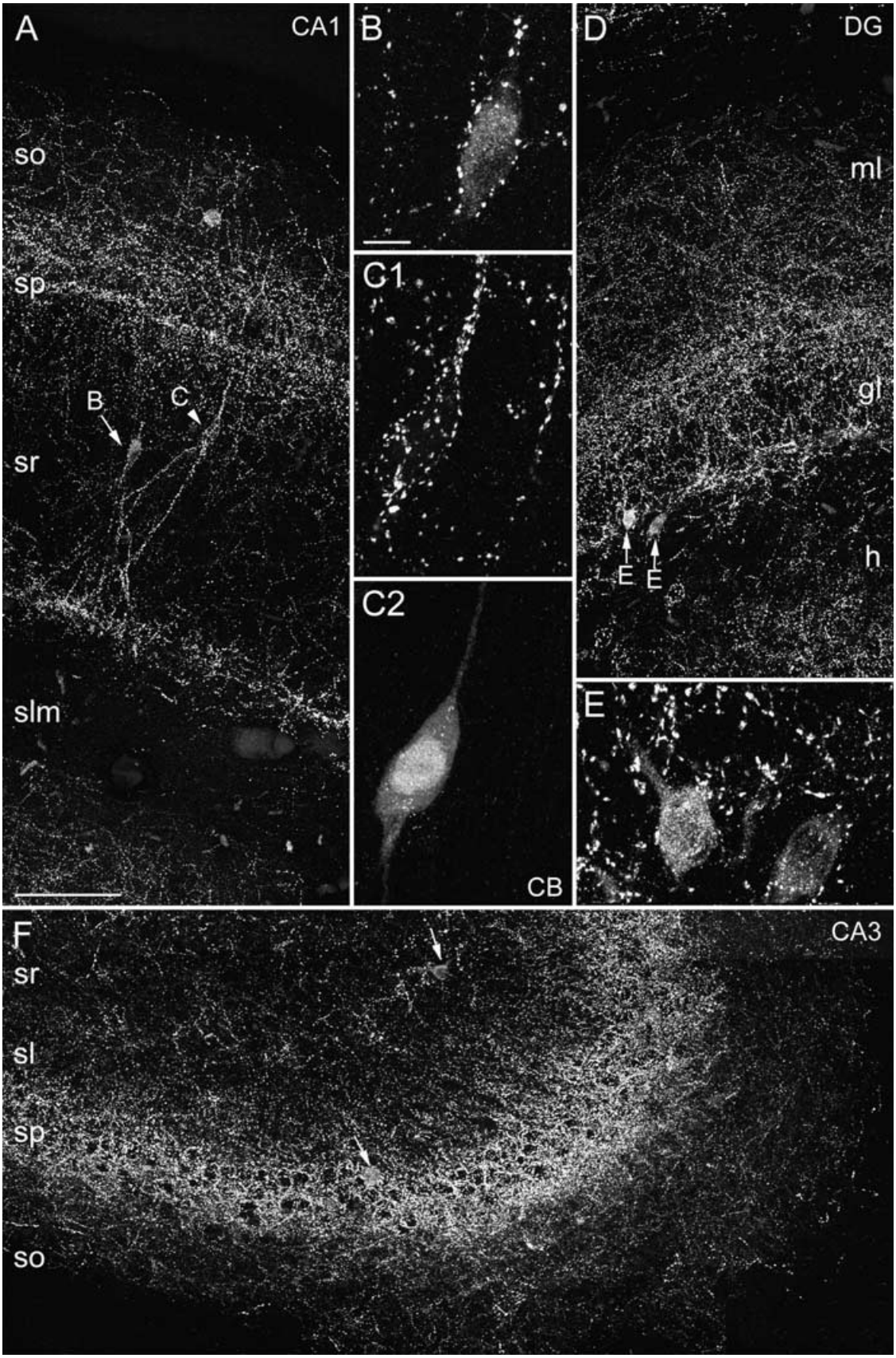
Distribution of VGLUT3 immunopositive somata and boutons

In the CA1 and CA3 areas (Fig. 2A and F) most VGLUT3 immunopositive somata were found in stratum (str.) radiatum (rad.); fewer were seen in strata pyramidale (pyr.) and oriens. For quantification, the very few labelled somata in str. lacunosum-moleculare (lac.-mol.) of the CA1 area were grouped with those in str. rad. (Table 2). Occasional VGLUT3-positive somata appeared in str. lucidum. In the dentate gyrus VGLUT3-positive somata were frequent in the subgranular zone, and some labelled somata occurred in the hilus (Fig. 2D and E).

Boutons immunopositive for VGLUT3 were seen in all hippocampal layers, albeit with different density. In the CA1 area (Fig. 2A), a narrow band of dense boutons was at the border of str. rad. and lac.-mol., and a wider one followed str. pyr. and adjacent part of the dendritic layers. The highest density of VGLUT3-positive boutons was found in str. pyr. of the CA3 area (Fig. 2F). In the CA1 and CA3 areas, VGLUT3-positive boutons outlined the soma and proximal dendrites of pyramidal cells and those of putative interneurons (Fig. 2A–C). In the dentate gyrus (Fig. 2D), immunoreactive boutons densely filled the granule cell layer and the inner molecular layer, whereas in the outer part of the molecular layer they were sparser. In the hilar region VGLUT3-positive boutons were frequently seen in close contact with soma and proximal dendrites of neurons (Fig. 2D and E).

In the parietal isocortex (Fig. 3), VGLUT3-positive somata were much sparser than in the hippocampal formation and most of them were in layers II–III. Very few somata were labelled in the deeper layers (Fig. 3A and C). The density of immunoreactive boutons varied amongst the layers. They were densest in layers II–III; scattered boutons were seen in layer V and VI with a little higher density at the border with layer IV. As in the hippocampus, VGLUT3-positive boutons targeted VGLUT3-labelled (Fig. 3B and C), as well as unlabelled cells following the contour of their soma and proximal dendrites. Some of these cells were positive for CB (Fig. 3D).

In all the studied regions, the VGLUT3-positive somata had the appearance of interneurons. The intensity of somatic immunoreactivity was variable from hardly detectable to as intense as in nerve terminals. The immunoreactivity also spread to the most proximal dendrites and faded with distance from the cell body. Such a pattern is suggestive of the presence of the protein in the endoplasmic reticulum at the site of synthesis, but a vesicular location cannot be excluded.


A
CA1
so
sp
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B
C1
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CB
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CA3
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TABLE 2. Subpopulations of CCK interneurons, which were colabelled for VGLUT3, CB, CR or VIP

		Immunoreaction								
		CCK/CB/VGLUT3*			CCK/CR/VGLUT3			CCK/VIP/VGLUT3		
Layer	Animal	CCK cells tested (*n*)	CB (%)	VGLUT3 (%)	CCK cells tested (*n*)	CR (%)	VGLUT3 (%)	CCK cells tested (*n*)	VIP (%)	VGLUT3 (%)
Str. lacunosum-moleculare and radiatum together	1	28	28.6	35.7	21	0.0	52.4	33	0.0	33.3
	2	56	25.0	32.1	29	0.0	37.9	39	10.3	75.8
	3	33	24.2	21.2	28	0.0	39.3	40	2.5	46.6
Total cells tested (*n*)		117			78			112		
Mean ± SEM			25.9 ± 1.3	29.7 ± 4.4		n.a.	43.2 ± 4.6		4.3 ± 3.1	51.9 ± 12.5
Str. pyramidale	1	15	6.7	6.7	10	0.0	20.0	16	18.8	0.0
	2	15	6.7	26.7	13	0.0	0.0	16	31.3	0.0
	3	21	23.8	33.3	9	0.0	22.2	19	15.8	36.5
Total cells tested (*n*)		51			32			51		
Mean ± SEM			12.4 ± 5.7	22.2 ± 8.0		n.a.	14.1 ± 7.1		21.9 ± 4.7	12.3 ± 12.3
Str. oriens	1	4	75.0	25.0	8	0.0	12.5	2	50.0	0.0
	2	7	0.0	14.3	5	0.0	20.0	9	11.1	0.0
	3	5	20.0	40.0	3	0.0	0.0	10	20.0	0.0
Total cells tested (*n*)		16			16			21		
Mean ± SEM			31.7 ± 22.4	26.4 ± 7.5		n.a.	10.8 ± 5.8		27.0 ± 11.8	n.a.
All layers together	1	47	25.5	25.5	39	0.0	35.9	51	7.8	21.6
	2	78	19.2	29.5	47	0.0	25.5	64	15.6	20.3
	3	59	23.7	27.1	40	0.0	32.5	69	8.7	26.1
Total cells tested (*n*)		184			126			184		
Mean ± SEM			22.8 ± 1.9	27.4 ± 1.1		n.a.	31.3 ± 3.1		10.7 ± 2.5	22.7 ± 1.8

Values are percentage of the total number of tested CCK-positive interneurons (*n*). No triple-labelled cells were found, except in labelling for CCK, CB and VGLUT3, where three cells out of 184 were triple labelled. SEM, standard error of the mean. *Each data set was obtained from sections processed in a single run of immunoreaction for the given triplet of molecules. n.a., not applicable.

Immunoreactivity for VGLUT3 was not detected in the soma and dendrites of pyramidal cells in the hippocampus and isocortex, or in the dentate granule cells.

VGLUT3 immunoreactive perikarya and some boutons are positive for GAD

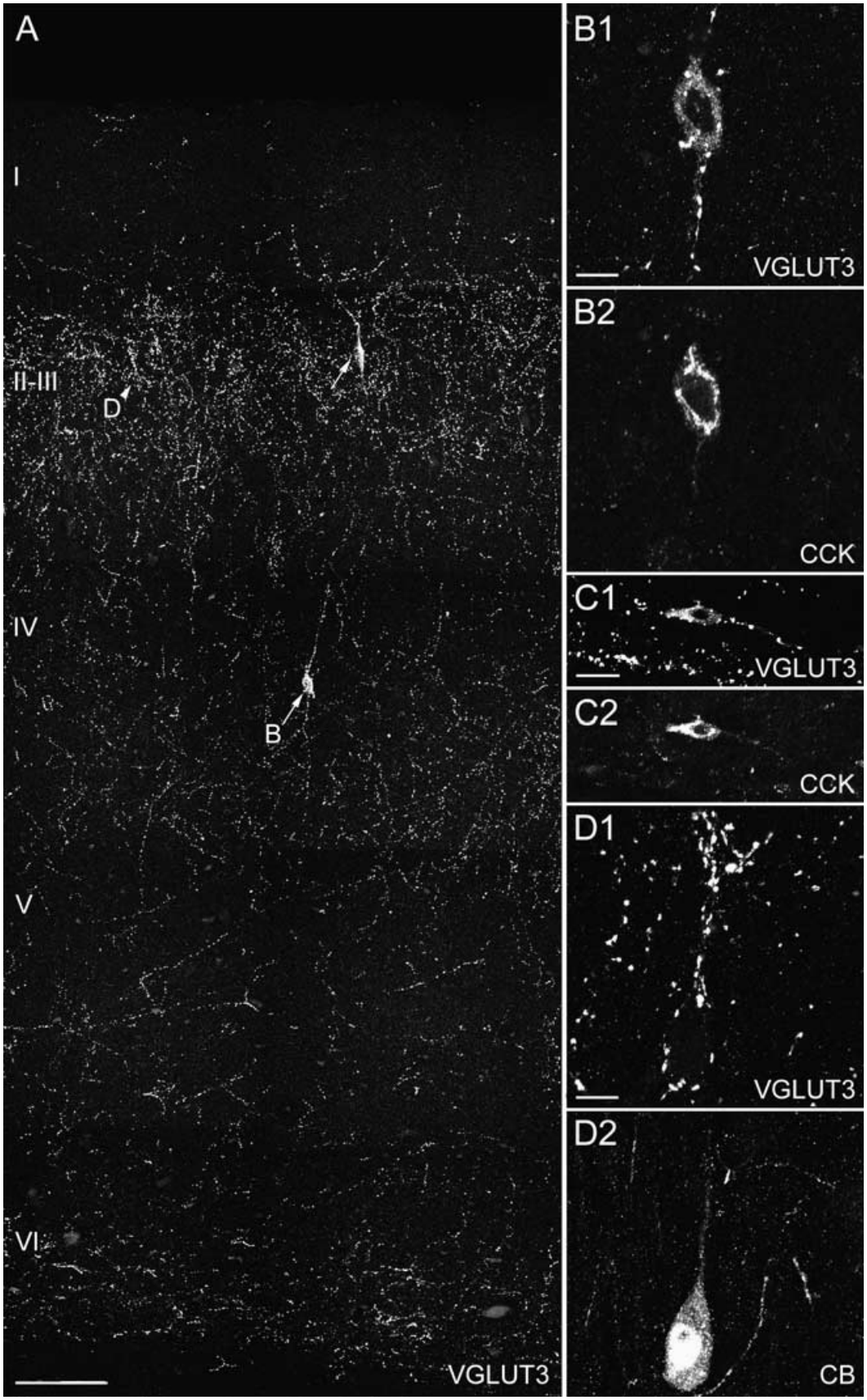
In agreement with previous results (Fremeau *et al*., 2002; Gras *et al*., 2002), VGLUT3 was found in the somata of GAD-expressing GABAergic interneurons (Fig. 4A and B). In boutons, however, the overlap varied; in the cell body layers most of them were double-labelled for VGLUT3 and GAD (Fig. 5A), whereas in the dendritic layers VGLUT3-positive boutons were mostly GAD-negative (Fig. 5B).

VGLUT3 immunopositive perikarya are positive for CCK but negative for CB

Most VGLUT3-positive cells were reported from str. rad. (Fremeau *et al*., 2002) where many interneurons express CB (Gulyas *et al*., 1991) and/or CCK (Cope *et al*., 2002). Therefore, we tested VGLUT3-positive somata for the presence of CCK and CB by triple immunofluorescence labelling and quantitative confocal microscopy. All VGLUT3-positive somata were immunoreactive for CCK (Fig. 4A–C), but not all CCK-positive somata were immunoreactive for VGLUT3 (Figs 4A and E, 5B, and 7A and B, and Table 2). Somata double-labelled for VGLUT3 and CCK were typically negative for CB (Fig. 4C and Table 2). CB-positive but CCK-negative somata were always VGLUT3-negative (Fig. 4D and E). Only three out of 184 tested VGLUT3- and CCK-positive cells were found also positive for CB. These triple-labelled somata were at the border of the CA1 and CA3 regions. On average, in three animals, across all layers in the CA1 area 26.8 ± 0.7% of CCK-positive interneurons were also labelled for VGLUT3, and another 22.8 ± 1.9% for CB (Table 2). This latter figure is in good agreement with earlier results (Cope *et al*., 2002; 20% and 30% in strata rad. and pyr., respectively). However, it is significantly higher than the corresponding 3% and 3.5% published by Gulyas *et al*. (1991). The difference may be due to better sampling by serial reconstruction and 3-D counting used in the current study (Coggeshall, 1992).

Boutons immunopositive for VGLUT3 innervated neurons double-labelled for VGLUT3 and CCK (Figs 2B, and 4A and C) and targeted even more heavily the somata and proximal dendrites of cells labelled for CB (Figs 2C, 4D and E, 8A, and 9B). In str. pyr. of the CA1 area, boutons colabelled for VGLUT3 and GAD were also positive for CCK (Fig. 5A). These triple labelled boutons were in close contact with the soma and proximal dendrites of principal cells and interneurons. In the

FIG. 2. Confocal microscopic images of sections immunolabelled with guinea pig antibody raised against VGLUT3. (A–C) In the CA1 area, VGLUT3-positive somata are present in str. oriens (so) and str. radiatum (sr). Both immunopositive (arrow and B) and some immunonegative cells (arrowhead and C) are heavily innervated by VGLUT3-positive boutons. A heavily innervated VGLUT3-negative interneuron soma (C1) is positive for CB (C2). Immunoreactive boutons are dense in the str. pyramidale (sp.) and in a band at the border of str. radiatum and lacunosum-moleculare (slm). (D–E) In the dentate gyrus (DG), VGLUT3-positive somata (arrows and E) are often located just below the granular layer (gl). The granular layer and the inner one-third of the molecular layer (ml) are particularly enriched in VGLUT3-positive boutons. (F) In the CA3 area, some interneuron somata in str. pyramidale (sp.) and radiatum are immunoreactive for VGLUT3 (arrows), and immunopositive boutons strongly innervate cells in str. pyramidale. sl; stratum lucidum. Scale bars, 100 μm A, D and F; 10 μm B, C and E.


A
I
II-III
IV
V
VI
D
B
VGLUT3
B1
VGLUT3
B2
CCK
C1
VGLUT3
C2
CCK
D1
VGLUT3
D2
CB

dendritic layers few VGLUT3-positive boutons contacting interneurons were immunopositive for GAD or CCK (Fig. 5B).

A similar pattern of somatic colabelling was found in the parietal isocortex, CA3 area and the dentate gyrus (Figs 3B and C, and 6A and B). In a semiquantitative assessment all tested VGLUT3-immunoreactive neurons in the hippocampus (69 cells) and in the parietal isocortex (24 cells) were immunoreactive for CCK. Conversely, of the CCK-immunopositive cells tested for VGLUT3 in the CA3 area (89 cells), the dentate gyrus (76 cells) and the isocortex (111 cells), 43.8%, 72.4% and 27.0%, respectively, were double labelled. None of the 66 and 27 VGLUT3-positive cells in the hippocampus and isocortex, respectively, was immunoreactive for CB, but they were heavily targeted by VGLUT3-positive boutons (Fig. 3C). Most perisomatic VGLUT3-immunoreactive boutons on principal cells in the CA3 area and dentate gyrus were colabelled for CCK and GAD (not shown).

Testing VGLUT3-positive interneurons and boutons for VIP- and CCK-immunoreactivity

Cortical CCK-expressing basket cells were reported to express either CCK alone or CCK and VIP together (Acsady *et al*., 1996; Freund & Buzsaki, 1996; Kubota & Kawaguchi, 1997). Because a large proportion of VGLUT3-positive terminals appear to form pericellular baskets, we tested VGLUT3-positive somata for the presence of CCK and VIP. On average, CCK and VIP immunoreactivity occurred alone in 44.6% and 32.8% of the examined perikarya, respectively, across all layers of the CA1 area. All VGLUT3-positive somata were negative for VIP, but $10.7 \pm 2.5\%$ of CCK-positive cells were also positive for VIP (Fig. 7A and Table 2). This figure is similar to that reported by Kosaka *et al*. (1985). In these reactions more than 20% of CCK-positive interneurons was also VGLUT3-positive in their perikarya (Table 2). None of the tested VGLUT3-positive neurons in the CA3 area/dentate gyrus (54 cells) and isocortex (24 cells) was immunoreactive for VIP. Some VIP-positive somata were contacted by VGLU3-positive terminals, especially in str. pyr. (not shown).

Testing VGLUT3-positive interneurons and boutons for CCK and CR immunoreactivity

Interneuron selective (IS) neurons form a distinct population and express either CR or VIP, or both (Acsady *et al*., 1996; Freund & Buzsaki, 1996; Gulyas *et al*., 1996). We tested CR and CCK-immunolabelling in VGLUT3-positive somata in the CA1 area. Somatic CCK- or CR-labelling occurred alone in 32.7% and 54.8% of the cells, respectively. There was no colocalization between CR and VGLUT3, or CR and CCK (Fig. 7B and Table 2). Likewise, none of the tested VGLUT3-positive cells in the CA3 area/dentate gyrus (123 cells) and isocortex (40 cells) was immunoreactive for CR. Moreover, CR-positive somata were contacted by VGLUT3-positive boutons to a variable extent.

VGLUT3 immunoreactive interneurons are immunonegative for SS, mGluR1α and PV.

None of the tested VGLUT3-positive neurons in the hippocampus (54 cells) and isocortex (20 cells) was immunoreactive for SS (Fig. 7C). Somata positive for SS were not seen in close contact with VGLUT3 terminals. Because, in addition to SS-positive cells, mGluR1α is expressed by several distinct classes of interneuron in all layers (Ferraguti *et al*., 2004), we tested its presence in VGLUT3-positive somata. Very few double-labelled cells were found in reactions for mGluR1α and VGLUT3 in the hippocampus (data not shown).

Cells expressing PV represent approximately half of the interneurons in the hippocampus (Kosaka *et al*., 1987) and some of them innervate the cell bodies of pyramidal cells, as VGLUT3-positive terminals do. To determine the relationship between PV and VGLUT3-positive neurons, double immunolabelling was used. In somata and boutons there was no overlap between PV and VGLUT3 immunoreactivity, regardless of their colabelling with GAD, in the CA1 area (Fig 7D and E). Similarly, none of the 112 VGLUT3-positive cells tallied in other hippocampal areas and the isocortex was immunoreactive for PV. Some VGLUT3-positive terminals occasionally contacted PV-positive somata (not shown).

Molecular characterization of non-GABAergic, VGLUT3-positive terminals

Dense, small caliber 5-HTT-positive fibres were seen in all the tested hippocampal and isocortical regions. In the CA1 area, the distribution was similar to that of 5-HT (Fig. 8). Co-localization of VGLUT3 and 5-HTT was rare and it occurred in small dots in 5-HTT-labelled fibres and varicosities (Fig. 8A and B). Likewise, labellings for VGLUT3 and 5-HT were mostly separate (Fig. 8C); however, colabelling was found in large boutons at the border between str. rad. and lac.-mol. (Fig. 8D) and around some interneurons in the isocortex. Multiple labelling for VGLUT3, VMAT2 and CB revealed that the distribution of boutons colabelled for VGLUT3 and VMAT2 was similar to that of VGLUT3 and 5-HT, except that the former appeared to colocalize more frequently (Fig. 9A and B). The VGLUT3 and VMAT2 double-labelled boutons contacted CB-positive interneuron cell bodies and dendrites in str. rad. and in the border region with str. lac.-mol. (Fig. 9B), as well as in the isocortex.

In boutons and somata, no colocalization was found between VGLUT3 and VAChT (Fig. 9C), mGluR7a and b (Fig. 10A–C), mGluR8a (Fig. 10D), mGluR8b and tyrosine hydroxylase (not shown). Immunoreactivity for both mGluR8a and mGluR8b were mostly present in str. oriens, and is subject of a detailed separate study (Dalezios *et al*., 2001; unpublished observation).

Discussion

We have shown that VGLUT3-positive neurons in the hippocampus and isocortex represent a specific subset of GABAergic basket cells, which are immunoreactive for CCK and innervate pyramidal cells, as well as some interneurons. A significant proportion of large VGLUT3-positive boutons belong to a subset of serotonergic fibres, which innervate CB-positive and other interneurons. In addition, there is a population of VGLUT3-positive boutons, which are immunonegative for GABAergic, serotonergic, other aminergic and cholinergic molecular cell markers and also innervate interneurons. The origin and molecular characteristics of these boutons remain to be established.

FIG. 3. Confocal microscopic images of sections from the parietal isocortex labelled for VGLUT3 and CCK or CB. (A) The density of VGLUT3-positive boutons is highest in layers II–III. Somata immunoreactive for VGLUT3 are in layers II–III and at the border of layers IV and V (arrows). Both immunopositive (arrows and B) and immunonegative (arrowhead and D) are targeted by VGLUT3-positive boutons. (B) A VGLUT3-positive cell in layers II–III is colabelled for CCK; VGLUT3-positive boutons innervate the soma and proximal dendrites. (C) A VGLUT3-positive cell in layer VI is colabelled with CCK and is contacted by VGLUT3-positive boutons on its soma and proximal dendrites. (D) A CB-positive interneuron in layers II–III is surrounded by VGLUT3-positive terminals. Scale bars, 100 μm A; 20 μm C, 10 μm B and D.

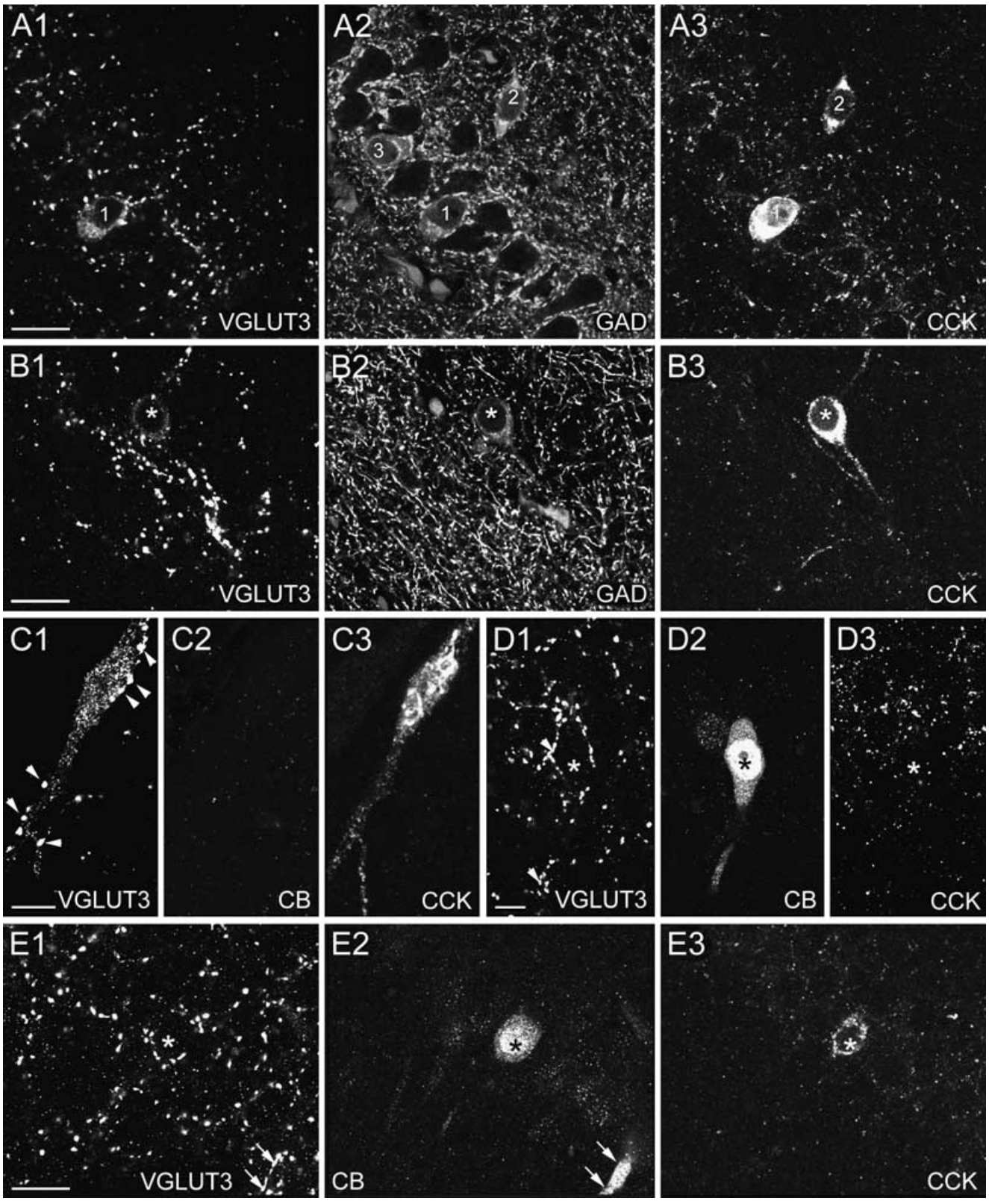


FIG. 4. Immunofluorescence labelling for VGLUT3, CCK and GAD or CB in the CA1 area. (A) An interneuron soma (1) is labelled for VGLUT3 (A1), GAD (A2) and CCK (A3), whereas soma 2 is labelled only for GAD and CCK. Soma 3 is immunopositive only for GAD, and pyramidal cell bodies are immunonegative for VGLUT3. (B) At the border between str. radiatum and lacunosum-moleculare, a soma (*) is immunopositive for VGLUT3 (B1), GAD (B2) and CCK (B3). (C) A soma in str. radiatum is labelled for VGLUT3 (C1) and CCK (C3), but not for CB (C2). The cell is heavily targeted by boutons positive for VGLUT3 (arrowheads in C1), which are immunonegative for CCK and CB. (D) In str. radiatum VGLUT3-positive boutons (arrowheads in D1) strongly innervate a CB-positive soma (*, D2) which is negative for VGLUT3 (D1) and CCK (D3). (E) In str. pyramidale, VGLUT3-positive boutons (E1) surround pyramidal somata and an interneuron soma (*), which is negative for VGLUT3, but positive for both CB (E2) and CCK (E3). A dendrite (arrows in E2), is positive for CB, and is strongly innervated by VGLUT3-positive boutons (arrows in E1). Scale bars, 20 μm A, B and E; 10 μm C and D.

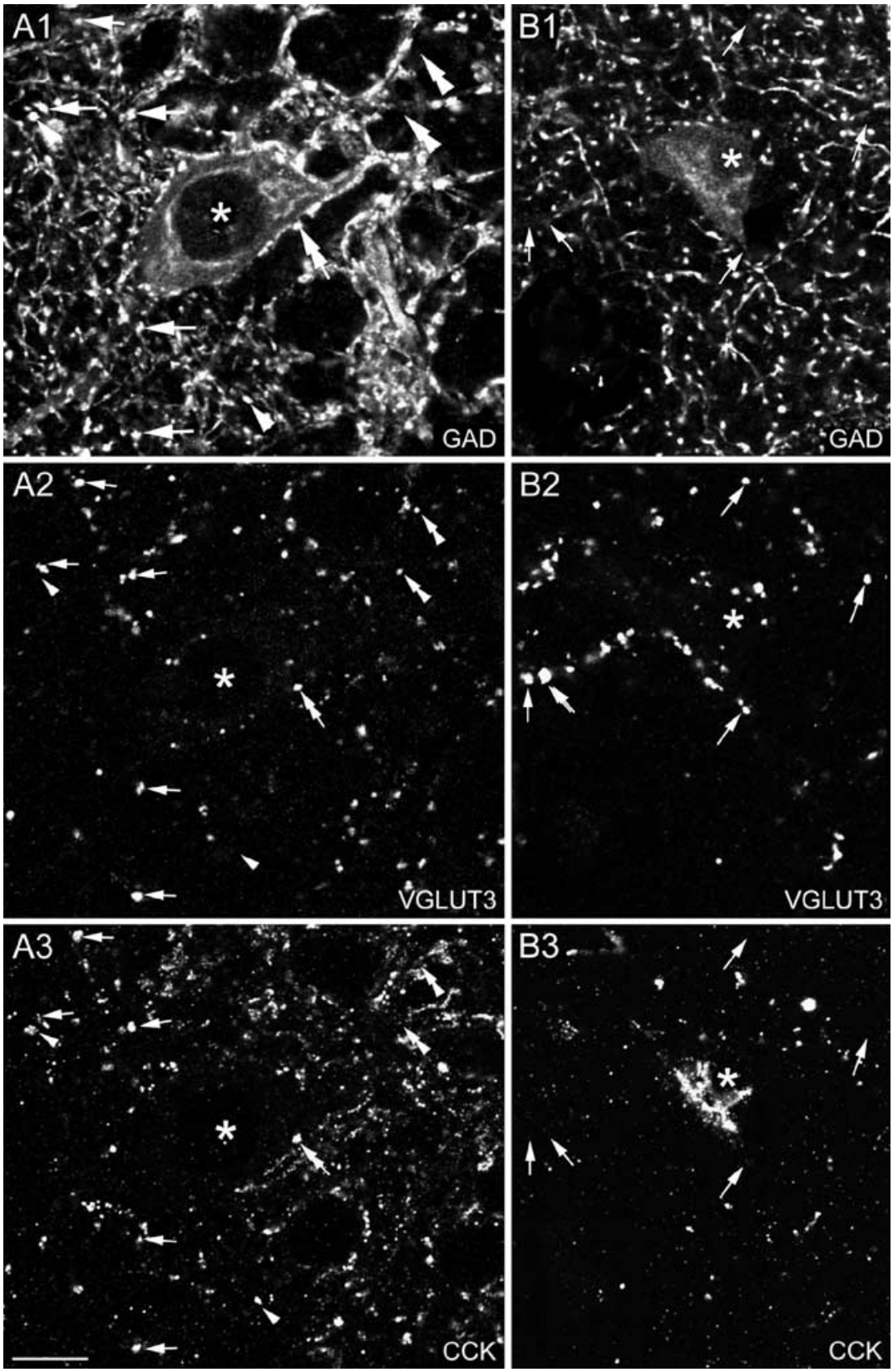


FIG. 5. Partial coexistence of VGLUT3, CCK and GAD immunolabelling in boutons of the CA1 area. (A) In str. pyramidale of the CA1 area, most boutons labelled for VGLUT3 are positive for CCK and for GAD (e.g. single arrows). However, some boutons are positive for VGLUT3 only (double arrowheads). Some CCK-labelled boutons are positive for GAD (e.g. arrowheads), but negative for VGLUT3. A GAD-positive soma (*) is contacted by boutons labelled for CCK, VGLUT3 and GAD (e.g. double arrow). (B) At the border of str. radiatum and lacunosum-moleculare, most VGLUT3 immunoreactive boutons were immunonegative for GAD and CCK (e.g. arrows). An interneuron (*) is immunopositive for CCK and GAD, but not for VGLUT3. Scale bar, 10 μm.

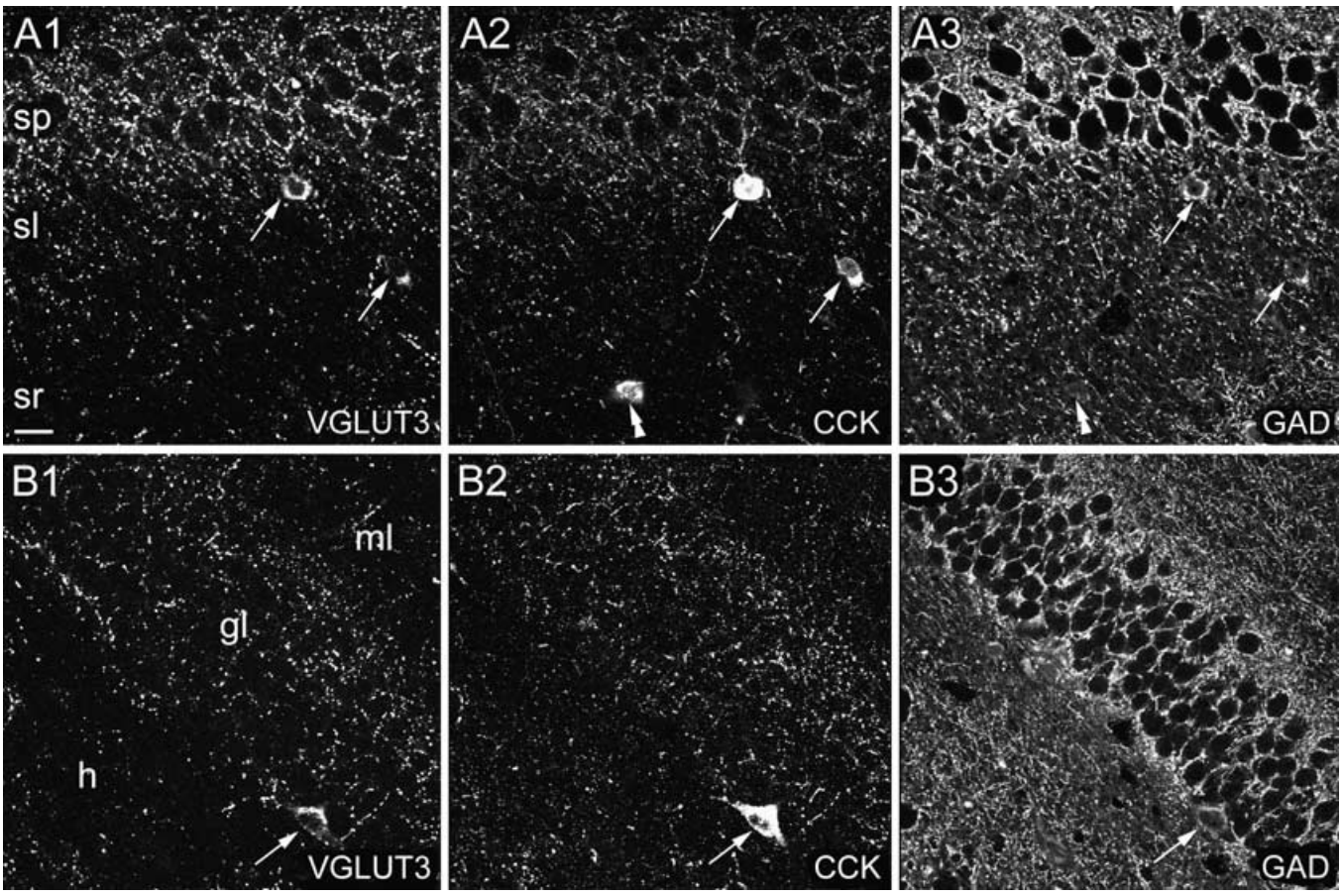
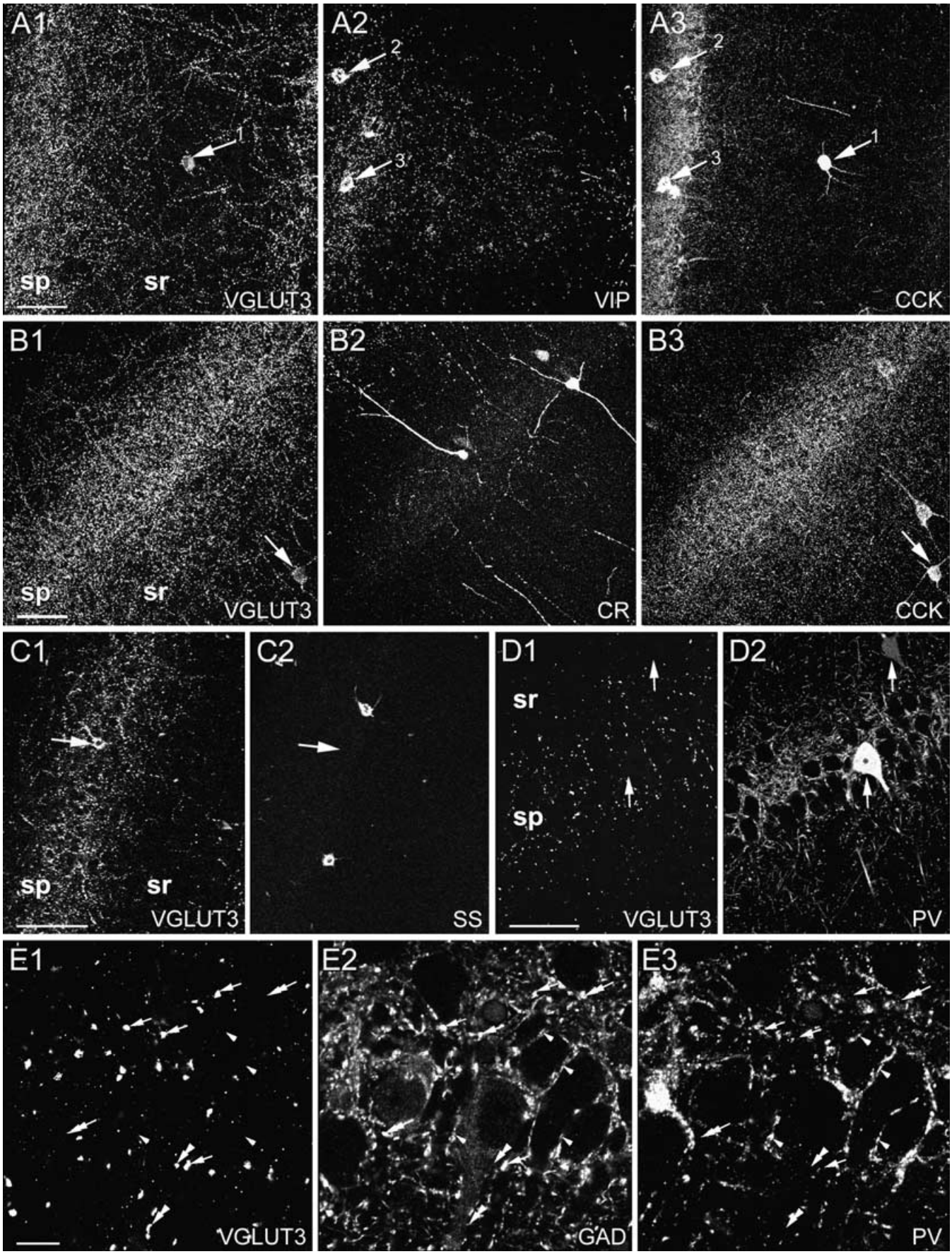


FIG. 6. Immunofluorescence labelling for VGLUT3, CCK and GAD in CA3 and dentate gyrus. (A) In str. lucidum (sl) and in the str. radiatum (sr), two somata are triple-labelled for VGLUT3, CCK and GAD (arrows), but another one (double arrowhead) is labelled only for CCK and GAD. Pyramidal cells are immunonegative for all three molecules and are surrounded by immunopositive boutons. (B) In the dentate gyrus the highest density of VGLUT3-positive boutons is in the granule cell layer (gl) and the inner molecular layer (ml); scattered boutons are present throughout the hilus (h). An interneuron soma (arrow) is labelled for VGLUT3, CCK and GAD. h; hilus, gl; granul cell layer, ml; molecular layer, sl; stratum lucidum, sp.; stratum pyramidale, sr; stratum radiatum. Scale bars, 20 μm.

VGLUT3 immunolabelling differentiates three types of basket cells

Basket cells are defined as innervating the somata and proximal dendrites of neurons (Ramon y Cajal, 1893; Lorente de No, 1934; Andersen *et al*., 1963; Szentagothai & Arbib, 1974). The best known population of these GABAergic cells is delineated by immunolabelling for PV (Katsumaru *et al*., 1988; Kawaguchi & Kubota, 1998), but this calcium binding protein is also expressed by other interneurons (Kawaguchi & Kubota, 1998; Pawelzik *et al*., 2002; Klausberger *et al*., 2003). The lack of PV immunoreactivity in VGLUT3-positive somata and boutons, the obligatory coexpression of VGLUT3 and CCK in interneuronal somata and the perisomatic location of boutons double-labelled for GAD and VGLUT3 in the principal cell layers demonstrate that VGLUT3 is present in a distinct type of basket cell. Some basket cells expressing CCK but negative for PV also express VIP (reviewed in Freund & Buzsaki, 1996). However, in our material, neither VIP-positive cell bodies nor boutons were positive for VGLUT3. Therefore, the CCK- and VGLUT3-positive cells constitute a novel subtype of basket cell. Approximately one-third of CCK-positive interneurons were also positive for VGLUT3. However, because of the relatively low level of somatic VGLUT3-immunoreactivity, the possibility of false negative somatic VGLUT3-labelling cannot be excluded. Therefore, this figure may be an underestimate. Indeed, the presence of CCK-positive interneurons immunonegative for all other tested molecular cell markers indicates that further immunocytochemical studies might change these proportions. More than one-fifth of CCK-positive interneurons also express CB, but they are unlikely to contribute to the basket cell populations (Cope *et al*., 2002). Moreover, VGLUT3-expressing somata were seldom colabelled for CB. The rare expression of mGluR1α in VGLUT3-positive cells and the complete lack of colabelling for CR and SS confirm the identity of VGLUT3-positive basket cells, as previous studies did not find these molecules in basket cells (Freund

FIG. 7. Immunofluorescence multiple labellings for VGLUT3 and VIP, CCK, CR, SS, PV and for VGLUT3, GAD and PV in the CA1 area. (A) An interneuron positive for VGLUT3 (1) is VIP negative, but positive for CCK. Interneurons (2 and 3) double labelled for VIP and CCK are negative for VGLUT3. (B) An interneuron is positive for both VGLUT3 and CCK but is negative for CR (arrow). (C) An interneuron is positive for VGLUT3 (arrow) but it is negative for SS. (D) Interneurons immunoreactive for PV (arrows) are negative for VGLUT3. (E) Boutons positive for VGLUT3 are negative for PV irrespective of the presence (e.g. arrows) or absence (e.g. double arrowheads) of GAD immunoreactivity. Some GAD-positive boutons are single-labelled (e.g. double arrows) and some others are colabelled for PV (e.g. arrowheads). sp.; str. pyramidale, sr; str. radiatum. Scale bars, 50 μm A, B, D; 100 μm C; 10 μm E.


A1
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CR
B3
CCK
C1
sp
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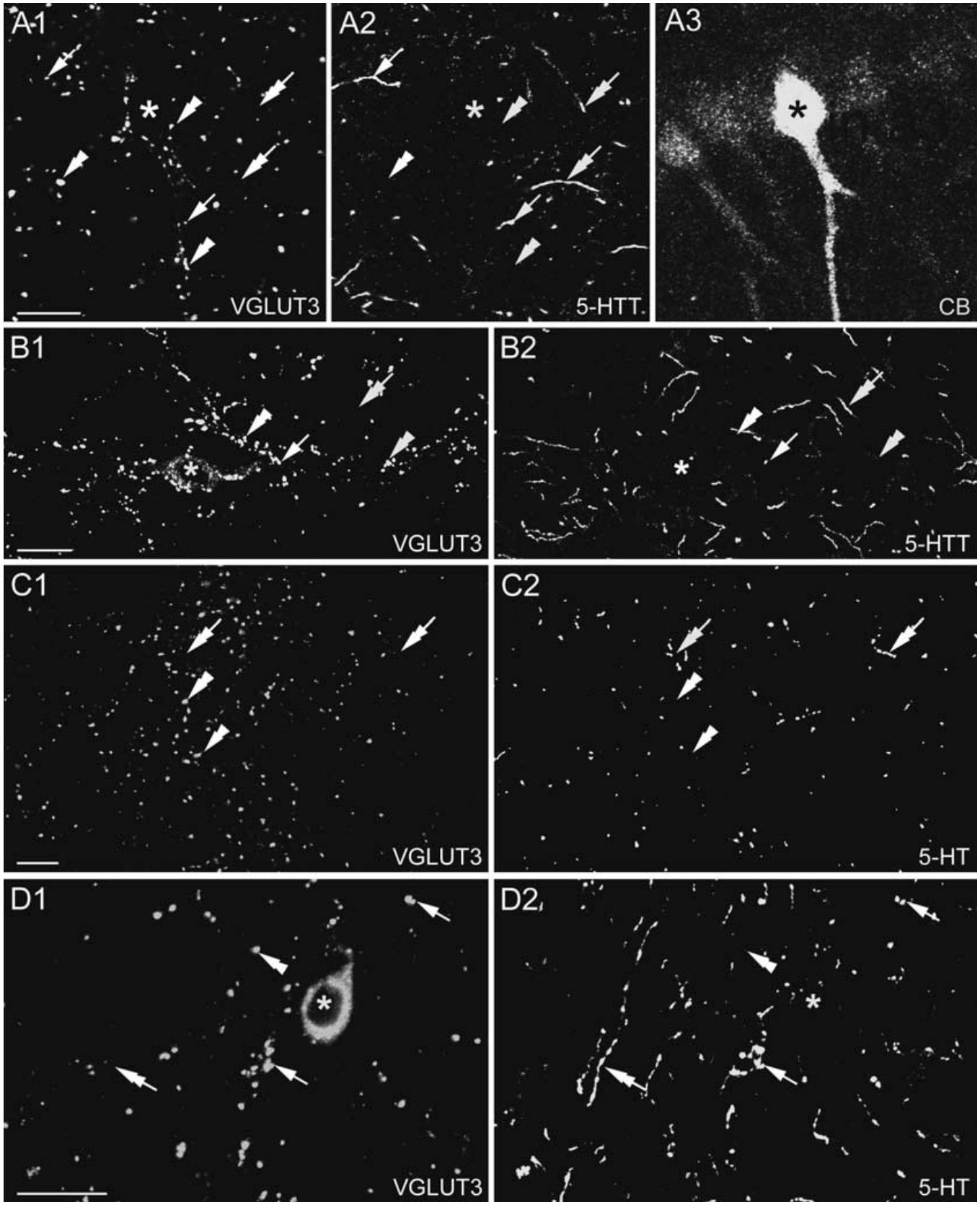
VGLUT3
C2
SS
D1
sr
sp
VGLUT3
D2
PV
E1
VGLUT3
E2
GAD
E3
PV

& Buzsaki, 1996; Ferraguti *et al*., 2004). Basket cells containing CCK are of particular interest because they express a high level of the cannabinoid receptor CB1 on their terminals and show a unique combination of molecular and physiological activity dependent mechanisms (Freund, 2003). The innervation of principal cells by three distinct classes of basket cell appears to be a general feature of the hippocampal formation and the supragranular layers of the isocortex.



Extrinsic afferents to the hippocampus expressing VGLUT3

In addition to the terminals of local GABAergic neurons, GABAergic afferents also innervate the hippocampus from the septum (Freund & Antal, 1988). These afferents terminate mainly on interneurons, but it is unlikely that they contain VGLUT3 for two reasons: (i) no mRNA expression for VGLUT3 was found in the medial septum (Gras *et al*., 2002; Schafer *et al*., 2002) and (ii) few, if any, VGLUT3 boutons were seen contacting PV-positive cells, which are a major target of septo-hippocampal terminals (Freund & Antal, 1988). Therefore, the perisomatic boutons in the pyramidal layer immunoreactive for VGLUT3 and GAD and/or CCK are very likely of local origin, as discussed above.

In the dendritic layers, however, most of the large boutons were negative for GAD, but positive for 5-HT or VMAT2, identifying them as serotonergic. Similar patterns were seen also in the isocortex. The serotonergic afferents to the hippocampus are of two types (Tork, 1990). One has thin fibres with small varicosities; the other has thick axons with large boutons. In our material, the antibody to 5-HTT labelled mainly the thin fibres including axons connecting the small varicosities, and revealed a dense web of fibres. Because of previous reports demonstrating the expression of VGLUT3 by serotonergic neurons in the raphe nuclei (Fremeau *et al*., 2002; Gras *et al*., 2002; Schafer *et al*., 2002), we expected the presence of VGLUT3-immunoreactivity in these fibres; but colabelling was relatively infrequent. This may be due to the small synaptic vesicle clusters in these *en passant* boutons, which therefore may be below the sensitivity of our detection. However, the large 5-HT- and VMAT2-positive boutons of thick caliber fibres were well-labelled for VGLUT3. The preferential target of these serotonergic inputs are the CB-containing interneurons (Freund *et al*., 1990), which were decorated by VMAT2-positive boutons colabelled with VGLUT3. In addition, these large boutons also decorated the VGLUT3-positive interneurons, which were colabelled for CCK as well. Previous studies demonstrated that some CCK-expressing cells are activated by 5-HT3 receptor mediated raphe-hippocampal input (Morales & Bloom, 1997). The present results suggest that the VGLUT3-expressing interneurons are amongst the targets of these afferents. The two raphe-hippocampal serotonergic fibre systems have different origins. The thin fibres are mainly from the dorsal raphe, whereas the large caliber ones originate from the median raphe nuclei (Kosofsky & Molliver, 1987). Stimulation of these ascending pathways effectively disrupts the theta activity in the hippocampus (reviewed in Vertes & Kocsis, 1997). The large boutons form well-defined synaptic junctions on the interneurons (Freund & Buzsaki, 1996), but it remains to be tested if these synaptic specializations contain excitatory amino acid receptors.

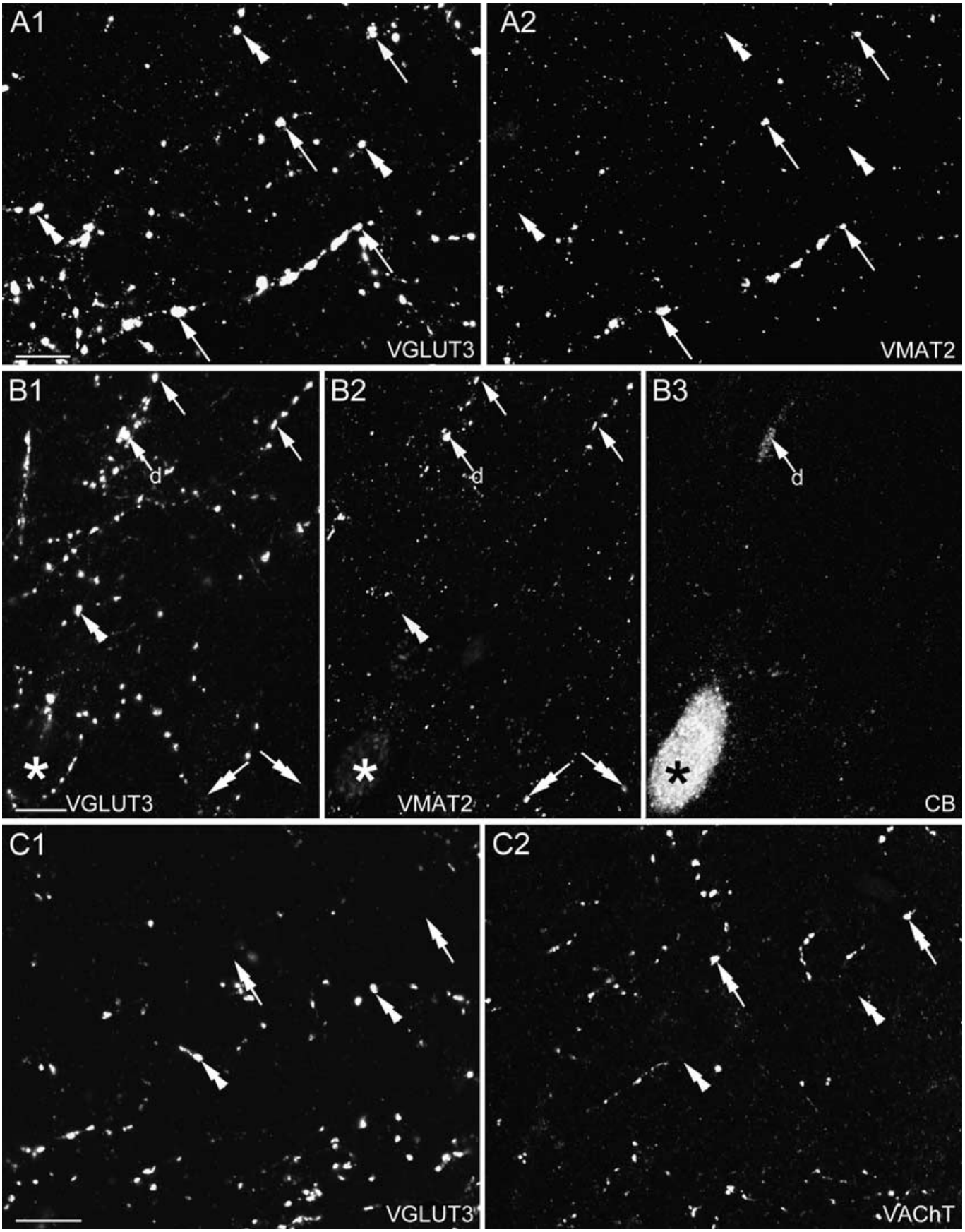
Some cholinergic neurons in the striatum, also have been shown to express VGLUT3 (Fremeau *et al*., 2002; Gras *et al*., 2002; Schafer *et al*., 2002) and the hippocampus is densely innervated by the cholinergic septo-hippocampal pathway, essential for generating theta activity (reviewed in Vertes & Kocsis, 1997). The lack of colabelling of VAChT with VGLUT3 in our study confirms previous findings reported in the hippocampus (Gras *et al*., 2002) and also in the isocortex (Schafer *et al*., 2002). This is in line with the absence of *in situ* hybridization signal for VGLUT3 in the medial septum and basal forebrain (Gras *et al*., 2002; Schafer *et al*., 2002), and shows that cholinergic neurons in the forebrain differ in VGLUT3 expression. Fibres immunopositive for tyrosine hydroxylase in the hippocampus did not show immunoreactivity for VGLUT3. This finding is in agreement with that obtained by Schafer *et al*. (2002). However, from our material, it is not clear whether there are too few vesicles in the catecholaminergic axons for our detection of VGLUT3, or the catecholaminergic fibres innervating the hippocampus genuinely lack this transporter. As VGLUT3 mRNA was not detected in dopaminergic and histaminergic neurons (Schafer *et al*., 2002), it is very likely that these afferents do not contribute to the VGLUT3-immunoreactivity described here, in the hippocampus and the isocortex.

A large proportion of VGLUT3-positive boutons, many of them innervating the same interneurons that received serotonergic innervation, remained unlabelled by all the tested antibodies in both the hippocampus and the isocortex. These boutons may also originate in the median raphe region, currently the only known nuclei to send such varicose fibres to CB-positive interneurons (Freund & Buzsaki, 1996) in the layers where we encountered these boutons. Although it is generally assumed that these are all serotonergic fibres and boutons, because similar boutons show serotonin immunoreactivity (Freund & Buzsaki, 1996), it has not been directly established that all afferents from the median raphe region are serotonergic and some may use different transmitters. In this respect it is noteworthy that in some VGLUT3-expressing cells of the medial raphe nuclei no serotonergic molecular cell markers could be demonstrated (Gras *et al*., 2002; Schafer *et al*., 2002). Alternatively, the boutons may originate from an as yet unknown neuronal population, which is unlikely to be GABAergic, because the boutons were GAD-negative.

VGLUT3-positive terminals lack mGluR7a,b and mGluR8a,b

If the substrate for VGLUT3, which is likely to be glutamate or a similar compound, is actually packaged and released together with GABA, it may act on pre- and/or postsynaptic glutamate receptors. Postsynaptic glutamate receptors have not been reported in GABAergic hippocampal synapses (Nusser *et al*., 1998). On the cell bodies of pyramidal cells, where many VGLUT3-positive terminals make synapses, only mGluR5 has been shown in low density at nonsynaptic sites (Lujan *et al*., 1996). However, several types of glutamate receptors are present on GABAergic axons in the hippocampus (Rodriguez-Moreno *et al*., 1997; Min *et al*., 1999; Poncer *et al*., 2000; Semyanov & Kullmann, 2000; Semyanov & Kullmann, 2001; Somogyi *et al*., 2003) and neocortex (Dalezios *et al*., 2002), and these may be activated by the excitatory amino acid potentially released from VGLUT3-positive GABAergic terminals. The apparent lack of colabelling between VGLUT3 and both splice variants of mGluR7 and mGluR8 in boutons makes it unlikely that the VGLUT3 substrate acts on these presynaptic receptors. Further experiments are necessary to establish if the VGLUT3/CCK-positive interneurons express presynaptic group II mGluRs and/or kainate receptors. Similarly the presence or absence of pre- and postsynaptic glutamate receptors has to be explored in the synapses made by VGLUT3-positive serotonergic terminals.

FIG. 8. Immunofluorescence multiple labellings for VGLUT3, 5-HTT, CB and 5-HT in the CA1 area. (A and B) In str. pyramidale (A) and at the border between str. radiatum and lacunosum-moleculare (B), VGLUT3-positive boutons are mostly negative for 5-HTT (e.g. double arrowheads) and conversely, fibres positive for 5-HTT are negative for VGLUT3 (e.g. double arrows). Rarely, VGLUT3-positive puncta are within 5-HTT-positive fibres (arrows). A VGLUT3-negative but strongly CB-positive interneuron (*) is amongst the weakly CB-positive pyramidal cells and is surrounded by VGLUT3-positive terminals (A1 and 3). (C) In str. pyramidale, VGLUT3-positive (e.g. double arrowheads) or 5-HT-positive (e.g. double arrows) boutons constitute separate populations. (D) At the border between str. radiatum and str. lacunosum-moleculare, some large boutons are immunopositive for both VGLUT3 and 5-HT (arrows), whereas others are labelled only for VGLUT3 (e.g. double arrowheads). Thin beaded fibres are 5-HT-positive but negative for VGLUT3 (double arrow). Scale bars, 20 μm.


A1
VGLUT3
A2
VMAT2
B1
d
VGLUT3
B2
d
VMAT2
B3
d
CB
C1
VGLUT3
C2
VAChT

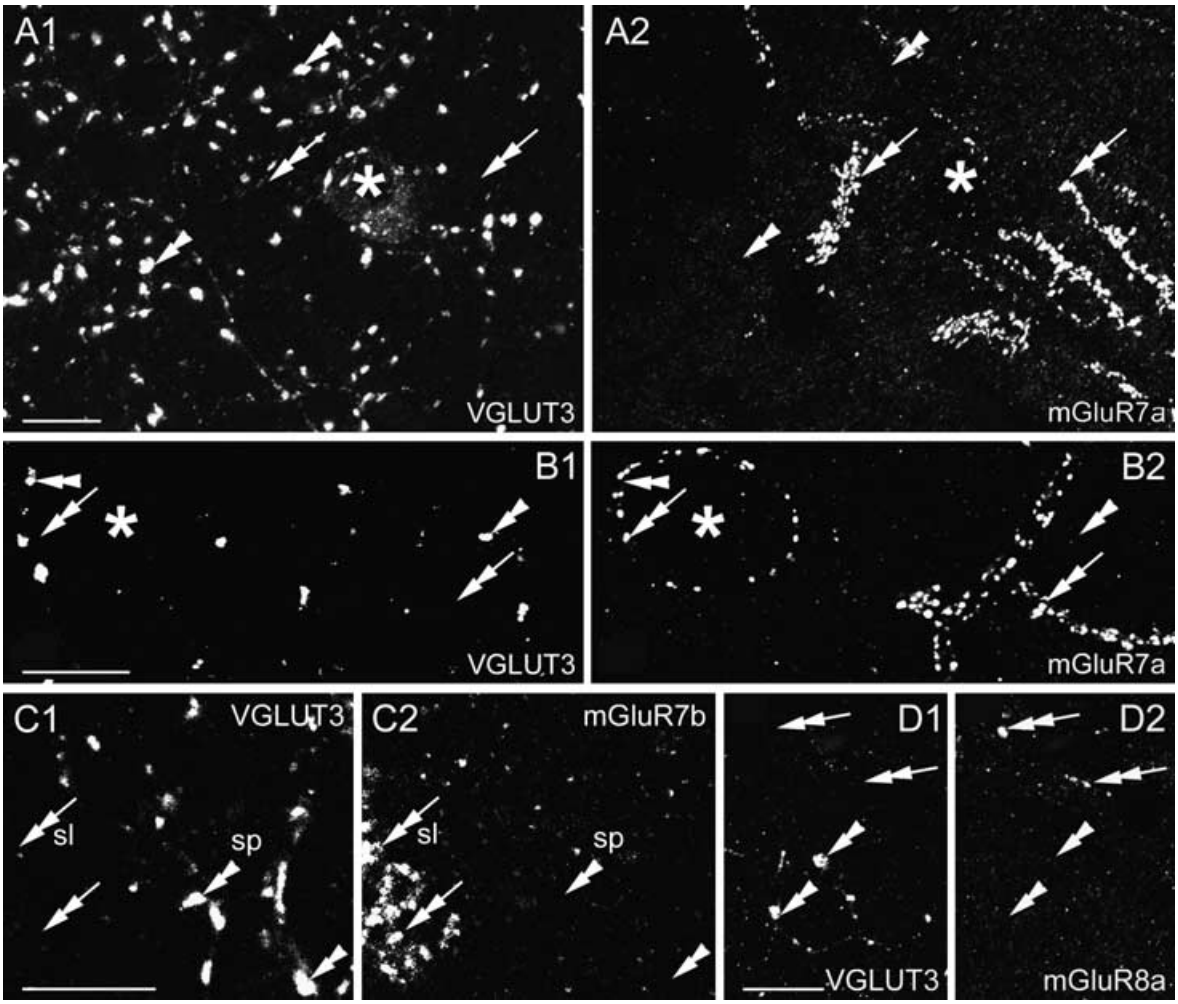


FIG. 10. Immunofluorescence double labelling for VGLUT3 and mGluR7a or b, or mGluR8a. (A) At the border between str. pyramidale and radiatum in the CA1 area, immunolabelling for VGLUT3 (double arrowheads) does not colocalize with mGluR7a labelling (double arrows). The VGLUT3-positive soma (*) is not innervated by mGluR7a-positive boutons. (B) The soma (*) and dendrites of an interneuron in str. oriens are heavily innervated by mGluR7a-positive boutons (double arrows) which are negative for VGLUT3. Boutons positive for VGLUT3 are negative for mGluR7a (double-arrow heads). (C) In the CA3 area, boutons are labelled either for VGLUT3 (double arrowheads) or for mGluR7b (double arrows). (D) In str. oriens of the CA1 area, immunolabelling for VGLUT3 (double arrowheads) does not colocalize with mGluR8a labelling (double arrows). sl; str. lucidum, sp.; str. pyramidale. Scale bars, 10 μm.

Possible functional significance of VGLUT3 in GABAergic and serotonergic synapses

It is unlikely that VGLUT3 is involved in the vesicular packaging of GABA or serotonin in terminals known or assumed to release these transmitters (Schafer *et al*., 2002; Takamori *et al*., 2002). This raises the possibility that the substrate of VGLUT3 is coreleased with the other transmitters. There is no direct information about the concentration of glutamate in CCK-positive basket cell terminals. Because CCK-positive interneurons (Somogyi *et al*., 1984; Kosaka *et al*., 1985) and their terminals (present study) are GAD-positive, their cytoplasmic glutamate level is likely to be low, as GAD converts glutamate to GABA. Nevertheless, postembedding immunohistochemistry has shown that significant levels of GABA and glutamate coexist in mossy fibre terminals of the hippocampus (Sandler & Smith, 1991; Szabo *et al*., 2000), in afferents to principal cells of the locus coeruleus (Somogyi & Llewellyn-Smith, 2001) and to motoneurons in the spinal cord (Somogyi, 2002). Moreover, synaptic vesicles immunoisolated on the basis of their GABA transporter content can accumulate significant amount of glutamate (Takamori *et al*., 2000b). The introduction of VGLUT1 into GABAergic neurons results in a mixed phenotype, i.e. cells that are capable of releasing both GABA and glutamate in single quanta (Takamori *et al*., 2000a). Furthermore, VGLUT3 is driven by the V-type H^+-ATPase, as are the other vesicular glutamate (Bellocchio *et al*., 2000; Takamori *et al*., 2001) and GABA/glycine (VIAAT; Takamori *et al*., 2002) transporters. Because both isomorphs of the mammalian vesicular monoamine transporter (VMAT1 and VMAT2) are operated by the same chemical force

FIG. 9. Immunofluorescence multiple labellings for VGLUT3, VMAT2, VAChT and CB in the CA1 area. (A and B) Most VMAT2-positive boutons are also positive for VGLUT3 (arrows) at the border of str. radiatum and lacunosum-moleculare (A) and in the middle of the str. radiatum (B). The majority of VGLUT3-positive boutons are VMAT2-negative (double arrowheads); only a few VMAT2-positive boutons are not labelled for VGLUT3 (double arrows). A CB-positive (*) cell body is contacted by boutons positive only for VGLUT3, whereas a CB-positive dendrite (d) is contacted by boutons double-labelled for VMAT2 and VGLUT3 (arrows) or VGLUT3 only. (C) None of the VGLUT3 boutons (e.g. double arrowheads) is colabelled for VAChT (double arrows) at the border of str. radiatum and str. lacunosum-moleculare. Scale bars, 10 μm.

(Gasnier, 2000), in principle, these transporters are compatible with each other and can operate in the same vesicular membrane. Therefore, a possibility of packaging and releasing transmitter mixtures cannot be excluded. Direct determination of glutamate levels in the VGLUT3-positive boutons could shed light on the possible involvement of glutamate in synaptic transmission of these terminals. Alternatively, VGLUT3 may be the sole transporter in a subset of vesicles, distinct from those storing GABA or serotonin within the same terminal. Further physiological and pharmacological studies are needed to clarify the synaptic interaction of two or more transmitters in the specific subsets of synapses characterized in the this study.

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Abbreviations

CB, calbindin D28K; CCK, cholecystokinin or pro-cholecystokinin; CR, calretinin; GAD, glutamic acid decarboxylase; GABA, γ-amino butyric acid; lac.-mol., lacunosum-moleculare; mGluR1α, metabotropic glutamate receptor type 1, splice variant α; mGluR7a, b, metabotropic glutamate receptor type 7, splice variant a and b; mGluR8a, b, metabotropic glutamate receptor type 8, splice variant a and b; PB, phosphate buffer; PV, parvalbumin; pyr., pyramidale; rad., radiatum; str., stratum; TBS, Tris buffered saline; TBSTx, Tris buffered saline containing Triton X-100; VGLUT3, vesicular glutamate transporter type 3; VIP, vasoactive intestinal polypeptide; VMAT2, vesicular monoamine transporter type 2; 5-HT, serotonin; 5-HTT, plasmalemmal serotonin transporter.

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