Enrichment of mGluR7a in the Presynaptic Active Zones of GABAergic and Non-GABAergic Terminals on Interneurons in the Rat Somatosensory Cortex

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The release of glutamate and GABA is modulated by presynaptic metabotropic glutamate receptors (mGluRs). We used immunocytochemical methods to define the location of the group III receptor mGluR7a in glutamatergic and GABAergic terminals innervating GABAergic interneurons and pyramidal cells. Immunoreactivity for mGluR7a was localized in the presynaptic active zone of both identified GABAergic and presumed glutamatergic terminals. Terminals innervating dendritic spines showed a variable level of receptor immunoreactivity, ranging from immunonegative to strongly immunopositive. The frequency of strongly mGluR7a positive terminals innervating the soma and dendrites of mGluR1α/somatostatin-expressing interneurons was very high relative to other neurons. On dendrites that received mGluR7a-enriched glutamatergic innervation, at least 80% of GABAergic terminals were immunopositive for mGluR7a. On such dendrites virtually all (95%) vasoactive intestinal polypeptide (VIP) positive (GABAergic) terminals were enriched in mGluR7a. The targets of VIP/mGluR7a-expressing terminals were mainly (88%) mGluR1α-expressing interneurons, which were mostly somatostatin immunopositive. Parvalbumin positive terminals were immunonegative for mGluR7a. Some parvalbumin immunoreactive dendrites received strongly mGluR7a positive terminals. The subcellular location, as well as the cell type and synapse-specific distribution of mGluR7a in isocortical neuronal circuits, is homologous to its distribution in the hippocampus. The specific location of mGluR7a in the presynaptic active zone of both glutamatergic and GABAergic synapses may be related to the proximity of calcium channels and the vesicle fusion machinery. The enrichment of mGluR7a in the main GABAergic, as well as in the glutamatergic, innervation of mGluR1α/somatostatin-expressing interneurons suggests that their activation is under unique regulation by extracellular glutamate.

Introduction

Glutamate is a major excitatory neurotransmitter in the mammalian cerebral cortex and its action is mediated by ionotropic and metabotropic receptors. The metabotropic glutamate receptors (mGluRs) are divided into three groups: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8), according to their molecular structure, pharmacological properties and their transduction mechanisms (Pin and Duvoisin, 1995; Cartmell and Schoepp, 2000). Group III mGluRs are located mostly presynaptically, and electrophysiological studies have shown that their activation mainly inhibits the release of glutamate from the presynaptic terminals [for a review, see Cartmell and Schoepp (Cartmell and Schoepp, 2000)]. There are several splice variants of some of the group III receptors, which are distributed differentially in the brain (Shigemoto et al., 1997; Corti et al., 1998; Kinoshita et al., 1998). One of the most abundant mGluRs in the cortex, mGluR7 (Ohishi et al., 1995; Corti et al., 1998;

Kinoshita et al., 1998; Kosinski et al., 1999), is negatively coupled to adenylate cyclase and requires a relatively high concentration of glutamate for activation (Wu et al., 1998; Cartmell and Schoepp, 2000).

Immunocytochemical studies have demonstrated the presence of mGluR7a or mGluR7b in both type I or 'asymmetrical', mostly glutamatergic, synapses (Bradley et al., 1996; Shigemoto et al., 1996, 1997), and in type II or 'symmetrical', mostly GABAergic, synapses (Shigemoto et al., 1997). Furthermore, in the islands of Calleja the presence of mGluR7a was directly shown in glutamate decarboxylase (GAD) immunopositive synaptic junctions by electron microscopic double immunolabelling (Kinoshita et al., 1998). The presence of mGluR7a in synapses at the presynaptic active zone of glutamatergic and GABAergic terminals indicates that it could act as an autoreceptor as well as a heteroreceptor regulating GABA release. Indeed, the selective group III receptor agonist, L-AP4, inhibits the release of GABA from cultured neurons (Schaffhauser et al., 1998; Lafon-Cazal et al., 1999), and in vivo activation of group III receptors by iontophoretically applied agonists can lead to disinhibition of responses evoked by natural stimulation in the somatosensory cortex (Wan and Cahusac, 1995). Furthermore, electrophysiological experiments in the thalamus (Salt and Eaton, 1995), hippocampus (Kogo et al., 1999; Semyanov and Kullmann, 2000), hypothalamus (van den Pol et al., 1998) and entorhinal cortex (Woodhall et al., 2001) show that presynaptic group III mGluRs depress GABA_A receptor-mediated postsynaptic responses. In line with the physiological studies, some of the isocortical GABAergic interneurons were found to express mRNA for mGluR7 (Cauli et al., 2000). However, there is no information about the presence of presynaptic mGluR7a on isocortical GABAergic axon terminals; therefore, we carried out an immunocytochemical analysis testing several synapse populations for the presence of mGluR7a.

There are multiple sources of glutamatergic innervation of neurons in the isocortex, and all tested pathways innervate both principal cells and several distinct types of cortical GABAergic cell. The latter show diversity in their molecular composition and input/output relationships supporting distinct roles in the cortical network (Somogyi et al., 1998). Therefore, potential presynaptic receptors influencing GABA release might have selective influence, depending not only on the cells that express them, but also on the postsynaptic targets of these cells. In the hippocampus, the main glutamatergic innervation of one type of GABAergic neuron, the mGluR1α/somatostatin-expressing interneuron, comes from local pyramidal cells (Blasco-Ibanez and Freund, 1995). Pyramidal cell axons make synapses with spines of other pyramidal cells and several distinct types of interneuron but, when they form synaptic junctions with an mGluR1α-

expressing cell, the synaptic terminal contains a much higher level of mGluR7a than synaptic terminals along the same axon innervating other cell types (Shigemoto *et al.*, 1996). The role of mGluR7a in synaptic transmission is not well understood, although it has been proposed that it may contribute to long-term depression of synaptic transmission in hippocampal interneurons (Laezza *et al.*, 1999). The mGluR1α/somatostatin-expressing cell occupies a specific place in the circuitry of the hippocampal formation because its GABAergic output is co-aligned with glutamatergic innervation of the distal dendrites of pyramidal cells from the entorhinal cortex (Maccaferri *et al.*, 2000).

At least some somatostatin-expressing cells in the isocortex show functional properties similar to the hippocampal neurons (Kawaguchi and Kubota, 1996; Reyes *et al.*, 1998; Kaiser *et al.*, 2001), but their place in the isocortical neuronal circuit remains undefined. The receptor subtype, mGluR1α, is also expressed in somatostatin immunopositive neurons throughout the cerebral cortex (Baude *et al.*, 1993; Stineholfer *et al.*, 2000). However, it is not known whether the mGluR1α-expressing neurons of the neocortex are innervated by terminals showing a selective enrichment in presynaptic mGluR7a. In preliminary light microscopic experiments, we noticed that in addition to a homogeneous neuropil labelling, more strongly mGluR7a immunoreactive dots outlined the somata and dendrites of some neurons in the isocortex resembling the hippocampal distribution. The aim of the present study was to establish if these dots corresponded to nerve terminals and to characterize the cells outlined by mGluR7a immunoreactivity. We investigated the presence of mGluR7a in different populations of non-GABAergic and GABAergic terminals. The results demonstrate a strong expression of presynaptic mGluR7a in both GABAergic and presumed glutamatergic terminals targeting mGluR1α/somatostatin-expressing, GABAergic interneurons. Some preliminary results have been published in abstract form (Dalezios *et al.*, 2001).

Materials and Methods

Tissue Preparation
Twelve adult (200–250 g) and two 15–17 days old male Wistar rats were deeply anaesthetized with Sagatal (pentobarbitone sodium, 60 mg/ml i.p.) and perfused transcardially first with 0.9% saline for 1 min. This was followed for ~15 min by freshly prepared fixative 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). After perfusion, the brains were removed from the skull and blocks of tissue were dissected and washed in 0.1 M PB for several hours. For electron microscopic immunocytochemistry, the blocks were incubated for cryoprotection in 10%, followed by 20% sucrose made up in PB at 4°C overnight. To increase the penetration of the reagents, the blocks were quickly frozen using liquid nitrogen and thawed in PB. The blocks were sectioned at 70 µm thickness on a vibratome. For light microscopy, the cryoprotection and freeze-thawing steps were omitted and 60 µm thick sections were cut.

Antibodies
Polyclonal antibodies were raised to mGluR7a in a rabbit to bacterial fusion protein containing amino acid residues 874–915 of rat mGluR7a (Shigemoto *et al.*, 1996). Immunoblot results and tests in heterologous expression systems indicated that the antibodies do not react with other mGluRs or proteins. No immunolabelling was obtained in the brain of mice deficient in mGluR7 protein (Sansig *et al.*, 2001). Affinity purified antibodies were used at a final protein concentration of 1 µg/ml.

Human antibodies to GAD, purified from the plasma of a patient suffering from Stiff-man syndrome (Oe *et al.*, 1996), were a gift from Dr K. Tanaka (Department of Neurology, Faculty of Medicine, Niigata University, Japan). They were used at 1 µg/ml protein concentration, and revealed immunolabelling in the isocortex, hippocampus and cerebellum identical to that published previously using different antibodies to GAD (Ribak, 1978; Mugnaini and Oertel, 1985).

Mouse monoclonal antibodies to synthetic porcine vasoactive intestinal polypeptide (VIP) were from two sources, which appear to derive from the same source originally raised by Dr J. Porter (University of Texas Health Science Center at Dallas). An early aliquot derived from East Acres Biologicals (Southbridge, MA); a more recent aliquot from Biogenesis (Poole, UK; cat. no. 9535-0504). Both aliquots were diluted to 1:5000. For specificity, the manufacturer refers to the paper of Dey *et al.* (1988), which reported results using immunocytochemistry and radioimmunoassay. Cortical immunolabelling under our conditions was identical to that reported using other antibodies [e.g. (Bayraktar *et al.*, 2000)].

Mouse monoclonal antibodies to parvalbumin (diluted 1:2000) were from Sigma (Poole, UK; Product No. P-3171) and in immunoblots are reported to label the expected band at 12 kDa. A monoclonal antibody to somatostatin (code: SOMA8, ascites fluid, a gift from Dr A. Buchan, Department of Physiology, University of British Columbia, Canada), characterized by Vincent *et al.* (1985) as recognizing somatostatin and SOM-28, was diluted 1:500.

Polyclonal antibodies to mGluR1α were a generous gift from Dr M. Watanabe and were used at a dilution of 1:700. They were raised in a guinea pig to residues 945–1127 of the mouse receptor sequence as described previously (Watanabe *et al.*, 1998; Tanaka *et al.*, 2000). The antibodies label a single protein band at 145 kDa in immunoblots of cerebellar membrane extracts. No membrane immunolabelling was obtained with this antibody in mGluR1-deficient mouse brain (Dr Francesco Ferraguti, MRC Laboratory, personal communication).

Immunocytochemistry for Light Microscopy
Floating sections were incubated in 20% normal goat serum (NGS) diluted in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl and 0.1% Triton X-100 (TBST) for 1 h. The sections were then incubated in a solution of primary antibodies diluted in TBST containing 1% NGS overnight at 4°C. After washes in TBST, the sections were incubated overnight at 4°C in a solution containing donkey anti-mouse 7-amino-4-methylcoumarin-3-acetic acid (AMCA, diluted 1:100; Jackson ImmunoResearch, West Grove, PA), anti-rabbit Alexa®-488 (diluted 1:1000; Molecular Probes, Leiden, The Netherlands) and donkey anti-guinea-pig Cy-3® (diluted 1:400; Jackson ImmunoResearch). After several washes in buffer the sections were mounted on slides, covered with Vectashield (Vector Labs, Burlingame, CA) under a coverslip. Immunofluorescence was studied using a Leica dichromatic mirror system and the A4 filter block [excitation filter, BP 360/40 nm, reflection short pass filter (RKP) 400 nm, suppression filter BP 470/40 nm] for visualizing AMCA, the L5 block (excitation filter, BP 480/40 nm, RKP 505 nm, suppression filter BP 527/30 nm) for recording Alexa®-488 fluorescence and the Y3 block (excitation filter, BP 545/30 nm, RKP 565 nm, suppression filter BP 610/75 nm) for recording Cy3 fluorescence. Cells were recorded on a CCD camera, analysed and displayed using the Openlab software (Improvision, Coventry, UK). Brightness and contrast were adjusted for the whole frame; no part of a frame was enhanced or modified in any way.

Immunocytochemistry for Electron Microscopy
Floating sections were first incubated for 1 h in 20% NGS diluted in 0.9% NaCl buffered with 50 mM Tris (pH 7.4, TBS). Sections were then incubated in a solution of a primary antibody or in a mixture of up to three antibodies diluted in TBS containing 1% NGS, at least overnight or for several days. When a single primary antibody was used, it was visualized either by an immunoperoxidase method or by silver-intensified immunogold reaction. When more than one primary antibody was used, one of them was visualized by immunoperoxidase reaction and the second, and in one combination also the third, by silver-intensified immunogold reaction. The two different epitopes visualized by silver-intensified immunogold reaction were on different types of cells; therefore, the signals could be clearly identified. After primary antibody incubation, the sections were incubated overnight at 4°C in one, or in a mixture, of the following antibodies: goat anti-rabbit and/or anti-guinea pig IgG (Fab fragment, diluted 1:100) coupled to 1.4 nm gold

(Nanoprobes Inc., Stony Brook, NY) and made up in TBS containing 1% NGS, and biotinylated goat anti-mouse, or goat anti-rabbit, or goat anti-human secondary antibodies (diluted 1:50, Vector Labs). After several washes in TBS, when required, the sections were washed in double-distilled water, followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes) for 8–12 min. Subsequently, the sections were incubated in avidin–biotinylated horseradish peroxidase complex (ABC, diluted 1:100, Vector Labs) in TBS overnight at 4°C and washed in Tris buffer (TB, pH ~7.4). Peroxidase was visualized with DAB (0.5 mg/ml) using 0.01% H_2O_2 as substrate for 5–10 min. After washes in TB and PB, the sections were treated with 2% OsO_4 in PB for 30–50 min, washed in PB and double-distilled water and contrasted in freshly prepared 1% uranyl-acetate for 40–50 min. They were then dehydrated in a series of ethanol and propylene oxide and embedded flat in epoxy resin (Durcupan ACM, Fluka, Sigma-Aldrich, Gillingham, UK) on slides. After the polymerization of the resin, selected small pieces of the sections from the somatosensory cortex were re-embedded in Durcupan blocks for sectioning. Serial 70–80 nm thick sections were collected on pioloform-coated copper grids. Electron microscopic sections from immunoperoxidase material were not lead contrasted; those containing only silver-intensified immunogold labelling were contrasted with lead citrate. Unless otherwise stated, electron microscopic samples were obtained from two different rat brains and two blocks of each animal were cut for electron microscopy.

Controls

To test method specificity in the procedures for light or electron microscopy, the primary antibody was omitted or replaced with 1% (v/v) normal serum of the species of the primary antibody. No selective labelling was observed. When double or triple labelling was used, some sections were always incubated with only one primary antibody and the full complement of secondary antibodies to test for any cross-reactivity of secondary antibodies. Other sections were incubated with two or three primary antibodies and one secondary antibody, followed by the full sequence of signal detection. No cross-labelling was detected that would influence the results. Specifically, using two or three primary antibodies, and both gold-labelled or biotinylated second antibodies, followed by the ABC complex and peroxidase reaction but no silver intensification, resulted only in amorphous horseradish peroxidase (HRP) end product and no metal particles were detected. Using the same sequence, but only silver intensification, without HRP reaction, produced silver granules, but no amorphous HRP end product. In other control experiments, silver-intensified gold reaction was used to detect a single primary antibody, but the full ABC sequence was also applied using a biotinylated second antibody that was raised to IgG from a species different from that of the applied primary antibody, followed by DAB reaction. Under these conditions, only infrequent small patches of HRP end product were detected, and the patches were not associated selectively with any particular cellular profile. In addition, the selective location of the signals in structures labelled only with one or the other of the signalling products within the same section, as well as having side by side double-labelled structures, showed that our procedures did not produce false-positive double-labelling results.

Statistical Analysis

Statistical differences between the frequencies of synaptic terminals labelled with a specific antibody were evaluated using the χ^2 test or Fisher's exact test when appropriate (Zar, 1999).

Results

Immunoreactivity for mGluR7a in the Presynaptic Active Zone of Glutamatergic and GABAergic Terminals

Our main interest was in the innervation of GABAergic neurons; therefore we focused the electron microscopic analysis on layers 2–4, where they have been extensively recorded and characterized (Kubota et al., 1994; Cauli et al., 1997; Kawaguchi and Kubota, 1997; Reyes et al., 1998; Gupta et al., 2000; Kaiser et al., 2001; Rozov et al., 2001; Sansig et al., 2001; Szabadics et al.,

2001; Tamas et al., 2002). Based on our light microscopic results, the pattern of receptor expression is not expected to be very different in the infragranular layers. In the isocortex, the dendrites of most GABAergic interneurons have few spines and receive the vast majority of their synaptic input on dendritic shafts. Therefore the dendritic shafts of interneurons are usually densely innervated by synaptic boutons making either type I or type II synapses (Szentagothai, 1975; Ribak, 1978; Somogyi et al., 1983; Kharazia et al., 1996). In contrast, the dendrites of pyramidal and spiny stellate cells emit numerous spines, which are the sites of termination for most of their synaptic inputs. The dendritic shafts of pyramidal cells receive mostly type II synaptic input (Feldman, 1984), but type I synapses also occur at low density (White and Hersch, 1981; Freund et al., 1985). It is, therefore, likely that dendritic shafts densely innervated mostly by type I synapses belong to GABAergic interneurons. Dendritic shafts receiving few synapses and examined only in a short segment could belong to pyramidal, spiny stellate or GABAergic cells.

In this study, at least 3 and up to 32 serial electron microscopic sections of each dendritic or axonal profile were assessed in order to evaluate the immunoreactivity for a given antibody. A profile was considered immunopositive if two independent experienced researchers agreed on this by inspecting images of serial sections. Weakly labelled profiles for receptors, e.g. labelled by one or two gold particles on the plasma membrane in one section, were considered immunopositive if this amount of labelling persisted in at least half, and not less than two, of the serial sections. Variability of labelling can arise as a result of variation in protein expression, the differential access of proteins to the antibodies depending on subcellular location, or on the location of the profile in the thick immunoreacted section. In an attempt to obtain biological information, we compared nearby profiles of different origin assuming that most of the difference would arise from differential protein expression. Nevertheless, variability arising from the differences in access to the antibody cannot be completely eliminated in the pre-embedding method. The post-embedding method, introduced earlier for receptor localization (Baude et al., 1993), could not be employed in the present study, because only a very weak signal has been obtained with the antibody to mGluR7a in our attempts so far.

We noticed that mGluR7a immunopositive terminals made either type I or type II synapses, as reported earlier in the hippocampus (Shigemoto et al., 1997). Most type II synapses in the isocortex are GABAergic. To test whether glutamatergic (type I) and GABAergic terminals express mGluR7a in the isocortex, we searched serial electron microscopic sections from the somatosensory cortex, which were double labelled for mGluR7a and GAD. One molecule was localized by silver-intensified immunogold and the other by immunoperoxidase reaction in the same section, and both molecules were visualized by both methods. Immunoreactivity for mGluR7a was present in many presynaptic terminals innervating dendritic spines presumably originating from pyramidal cells (Fig. 1A,B). The degree of presynaptic labelling was highly variable from spine to spine (Fig. 1A) as seen in the immunogold reaction; many synapses were immunonegative. The gold/silver particles showing mGluR7a immunoreactivity were mostly confined to the presynaptic active zone in the terminal (Fig. 1A). Occasionally, single particles were present in the presynaptic terminals over the synaptic vesicles. These may represent non-specific background labelling or receptors in intraterminal membranes; due to the scarcity of particles we were unable to distinguish
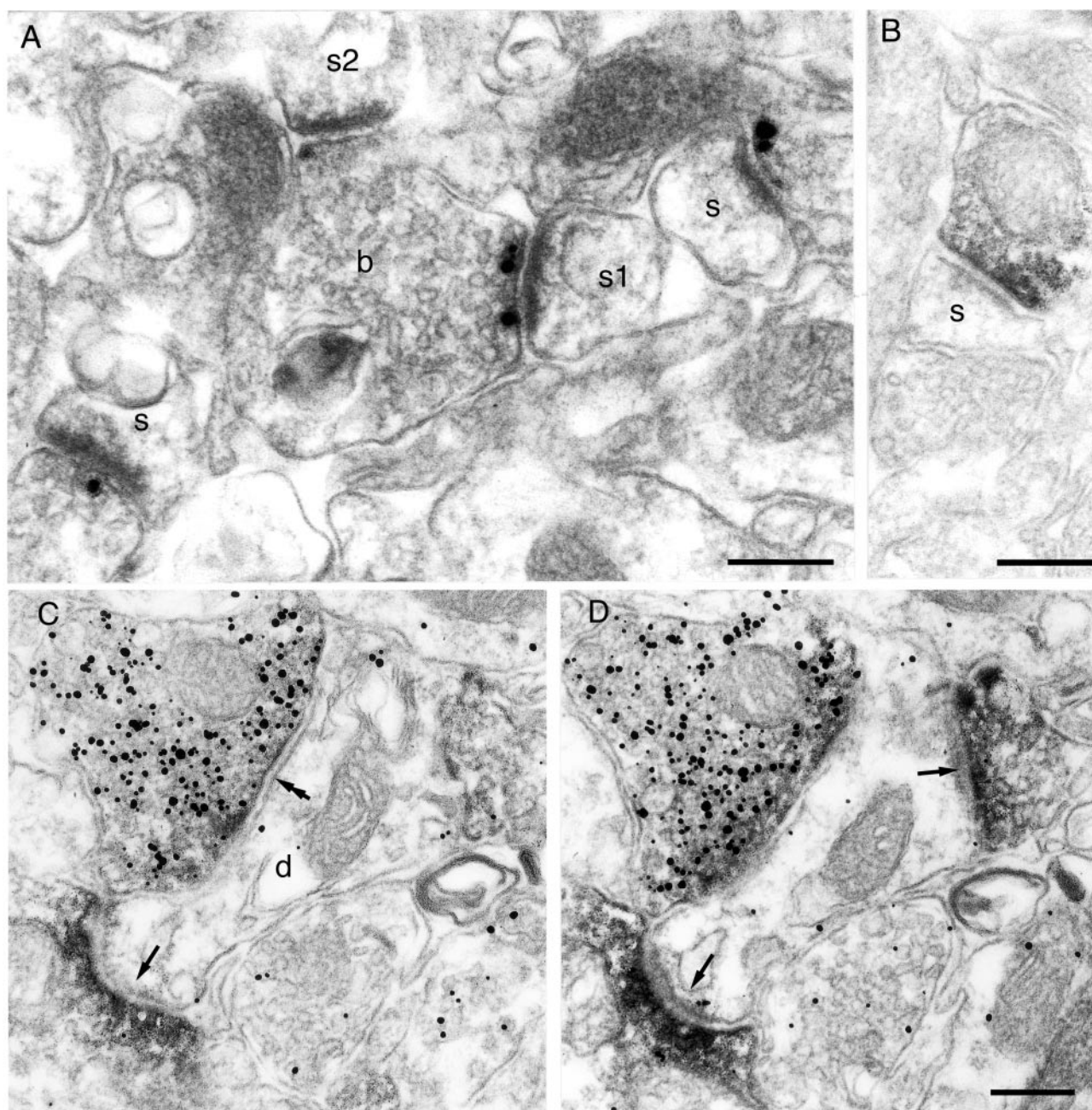
Figure 1. Both GABAergic and non-GABAergic synaptic boutons express mGluR7a in layer III. (*A*, *B*) Electron micrographs demonstrating mGluR7a immunolabelling (*A*, silver-intensified immunogold reaction; *B*, peroxidase reaction) in the presynaptic active zone of terminals making synapses with dendritic spines (s) presumably originating from pyramidal cells. The degree of immunoreactivity varies from bouton to bouton; one of them (b) makes synapses with two spines (s1, s2). Only the active zone of the synapse to s1 is immunopositive. The immunoperoxidase product seen in (*B*) spreads from the active zone into the terminal. (*C*, *D*) Serial sections showing a GAD immunopositive terminal (gold/silver particles) making a type II synapse (double arrows) on a dendritic profile. The same terminal is immunopositive for mGluR7a in the presynaptic active zone (peroxidase labelling). Two more terminals, which are immunonegative for GAD, make type I synapses (arrows) on the same dendritic profile indicating that this dendritic profile belongs to an interneuron. The peroxidase reaction product is most dense at the presynaptic active zone, but it also spreads among the vesicles. Scale bars: 0.2 μm.

between these possibilities. Some postsynaptic profiles, mostly dendrites and somata, also contained a low density of particles within the profile, which may represent receptors in internal membrane, but this could not be differentiated from background labelling. Particles were very rarely associated with dendritic and somatic plasma membranes or glial membranes. These particles may represent receptor labelling, but due to the inconsistency of the signal we could not clarify this. The immunoperoxidase reaction product for mGluR7a was mainly found in the presynaptic active zone, from where it diffused into the terminal, labelling the synaptic vesicles to a variable degree (Fig. 1*B–D*).

The degree of presynaptic labelling for mGluR7a was also highly variable on dendritic shafts, as seen from the number of immunoparticles, or from the presence or absence of immunoperoxidase product; many synapses were immuno-negative. However, on some dendritic shafts most synapses were highly immunopositive for mGluR7a (Fig. 1*C,D*). Dendritic shaft
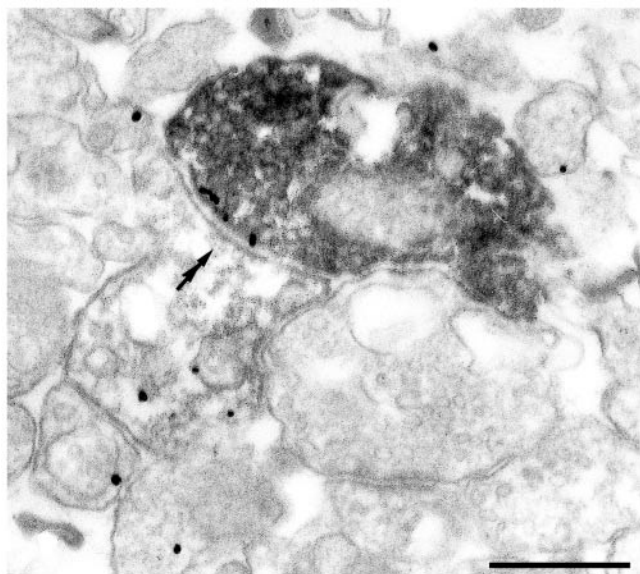
Figure 2. Presence of mGluR7a (gold/silver particles) in the presynaptic active zone of a GABAergic terminal labelled for GAD (peroxidase reaction). The terminal makes a type II synapse (double arrows) with a dendritic profile. Immunoreactivity for mGluR7a in the terminal is confined to the presynaptic active zone. Scale bar: 0.5 μm.

profiles receiving synaptic input enriched in presynaptically located mGluR7a were identified in cortical layers I–IV. Such dendrites were rare in any given electron microscopic section, and our initial attempt to sample them randomly was not successful, given the manpower available for this project. Therefore, we introduced a targeted sampling strategy in order to evaluate the GABAergic input of a subpopulation of dendrites, which could be identified as innervated by mGluR7a positive terminals. We included in the sample every dendritic profile encountered during the search of a given section that received at least one axon terminal showing GAD and one terminal showing mGluR7a immunoreactivity. This was necessary, because we were particularly interested in the receptor expression of the GABAergic terminals that are a minority of presynaptic boutons on any dendritic shaft. Furthermore, because we used a pre-embedding immunoreaction, which might result in differential antibody penetration and false negative results, special care was taken to sample at a depth of tissue where we could detect immunoreactivity resulting from labelling by both primary antibodies. We also counted the synaptic boutons on each profile and classified them into four categories according to their immunoreactivity: (i) only GAD positive, (ii) only mGluR7a positive (Fig. 1), (iii) double labelled for both GAD and mGluR7a (Figs 1 and 2) and (iv) unlabelled boutons.

Immunoreactivity for GAD was visualized by either per-oxidase product (Fig. 2) or by silver-intensified immunogold reaction (Fig. 1). In both cases, the whole cytoplasmic region of the terminals was uniformly labelled. The peroxidase and gold/silver signals could clearly be differentiated in boutons that showed labelling for both GAD and mGluR7a (Figs 1 and 2). The antibodies to GAD also labelled somata and dendritic shafts infrequently. In these structures the density of the peroxidase product or the gold/silver particles was much lower than that in synaptic boutons, and it is likely that under our conditions most dendrites originating from GABAergic neurons remained unlabelled.

A total of 58 dendritic profiles were analysed. Twenty-eight dendritic profiles (48%) were found to receive at least one

double-labelled GABAergic axon terminal making a type II synapse as well as type I synapses from terminals labelled only for mGluR7a. Another 23 dendritic profiles (40%) received at least one type II synapse from a double-labelled terminal and unlabelled type I synapses. Only seven (12%) of the dendritic profiles which received type I synapses from terminals positive for mGluR7a received GABAergic synapses that were not labelled for mGluR7a. Thus, 88% of mGluR7a-decorated dendritic profiles, selected according to the criteria described above, received GABAergic synapses, which expressed mGluR7a. These data suggest that the synaptic release sites of one or more subpopulations of GABAergic interneuron innervating other interneurons are enriched in mGluR7a. Among the most probable candidates for the presynaptic cells are the VIP/calretinin-expressing neurons (Acsady *et al.*, 1996; Meskenaite, 1997; Cauli *et al.*, 2000). Therefore, we used double-labelling immunocytochemistry for VIP and mGluR7a to characterize the GABAergic neurons expressing mGluR7a at their presynaptic active zone.

VIP Immunopositive Axon Terminals Express mGluR7a

Dendritic profiles receiving synapses from at least one axon terminal showing VIP (immunoperoxidase) and one terminal showing mGluR7a (gold/silver precipitate) immunoreactivity were recorded (*n* = 47, Fig. 3). The targeted selection criterion was necessary to sample sufficient number of terminals; it also resulted in a lack of information about VIP positive terminals targeting other cells. Axon terminals (peroxidase product) immunopositive for VIP made type II synapses on mGluR7a-decorated dendrites, and were often immunopositive for mGluR7a in their presynaptic active zone. The number and density of gold/silver particles in the VIP positive synaptic terminals did not appear to differ from those of the type I synapses on the same dendrites, indicating that the amount of mGluR7a is similar in glutamatergic and VIP positive (GABAergic) synapses innervating these dendrites. Forty (85%) of the dendrites received at least one double-labelled terminal as well as type I synapses immunopositive for mGluR7a. Six dendritic profiles (12.8%) received one or two double-labelled terminals, but no other terminals positive for mGluR7a. Only one dendritic profile (2.2%) received a VIP positive terminal that was not labelled for mGluR7a, although the same dendrite was the synaptic target of another mGluR7a positive bouton. Thus, virtually all the VIP positive axon terminals, which give synapses to interneuron dendrites decorated by mGluR7a-expressing terminals, also express mGluR7a. In this sample, we did not encounter VIP positive axon terminals targeting VIP positive dendrites. Next, we explored the identity of interneurons innervated by mGluR7a-enriched terminals.

Cortical Somatostatin/mGluR1α-expressing Neurons Are Targets of Synaptic Boutons Enriched in mGluR7a

In hippocampus, axon terminals expressing mGluR7a surround the dendrites of mGluR1α-expressing GABAergic neurons (Shigemoto *et al.*, 1996), many of which also express somato-statin (Baude *et al.*, 1993; Hampson *et al.*, 1994). The majority of isocortical mGluR1α-expressing GABAergic neurons also express somatostatin (Baude *et al.*, 1993; Stinehelfer *et al.*, 2000). However, it has not been reported whether these neurons are the targets of synaptic terminals enriched in mGluR7a. Triple immunofluorescence experiments were carried out with antibodies specific for somatostatin, mGluR1α and mGluR7a. As expected, somatostatin positive neurons both in young and in adult rats were densely labelled for mGluR1α and received
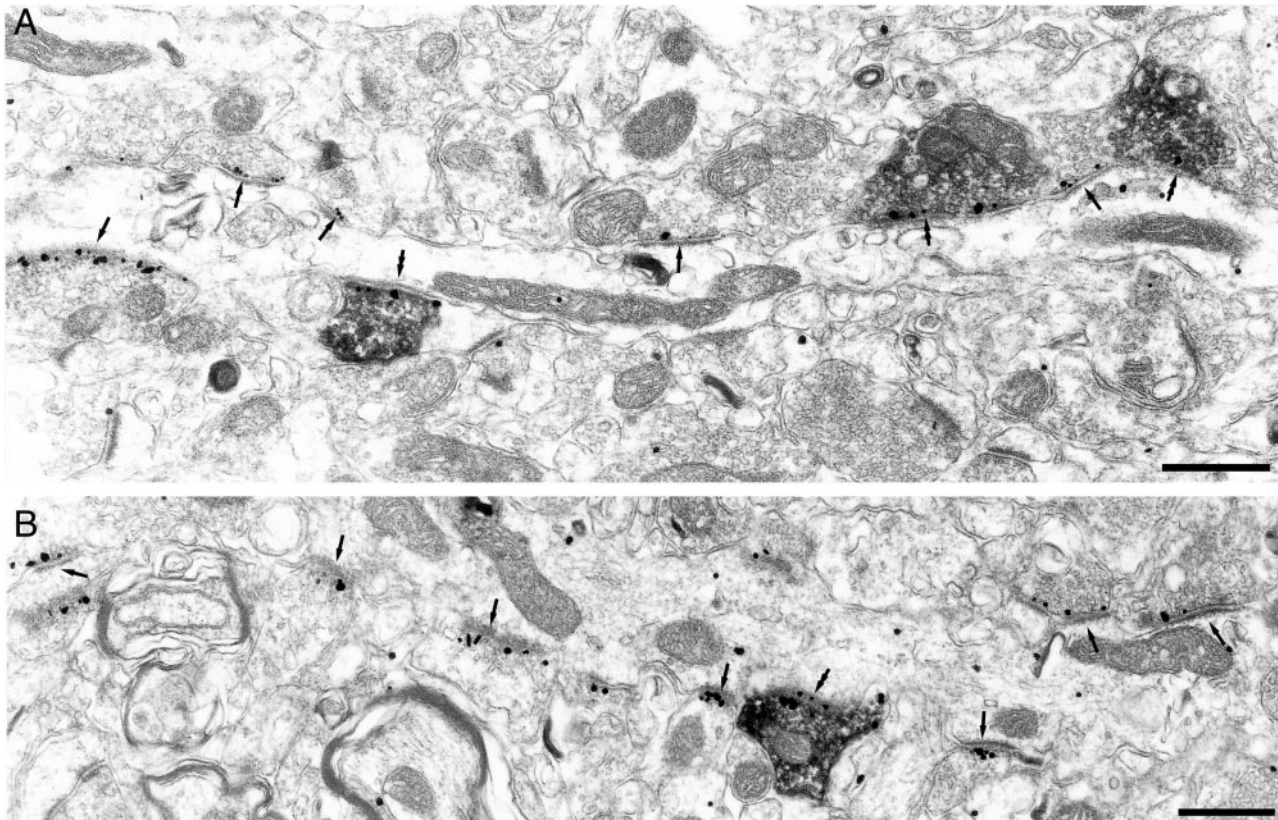
Figure 3. (*A, B*) Electron micrographs showing VIP immunopositive synaptic boutons (peroxidase labelling) making type II synapses on two dendritic shafts (double arrows). The VIP positive boutons are immunoreactive for mGluR7a at their presynaptic active zone (gold/silver particles). Numerous other boutons making type I synapses are also immunopositive for mGluR7a at their presynaptic active zone (arrows). Note that the VIP positive boutons have as many, or occasionally more, gold/silver particles as the presumed glutamatergic boutons. Scale bars: 0.5 μm.

terminals strongly enriched in mGluR7a in cortical layers 2–6 (Fig. 4). As reported in the hippocampus (Shigemoto *et al.*, 1996), the vast majority of mGluR1α immunopositive cells and dendrites were decorated by mGluR7a labelling, and the majority of strongly mGluR1α positive dendrites could be traced to somatostatin-expressing cells. Therefore, it is very likely that many of the mGluR7a/GAD positive terminals that we found to innervate interneuron dendrites, innervated somatostatin/mGluR1α-expressing GABAergic neurons. We have shown above that many isocortical VIP positive terminals express mGluR7a and innervate interneuron dendrites. Therefore, we tested whether VIP positive terminals target mGluR1α-expressing dendrites.

VIP Positive Axon Terminals Enriched in mGluR7a Target Dendrites Expressing mGluR1α

Electron microscopic, triple-labelling immunocytochemistry was applied to characterize the targets of the mGluR7a-enriched VIP positive axon terminals. Immunoperoxidase labelling was used for detecting VIP immunoreactivity and silver-intensified immunogold reaction for visualizing mGluR7a and mGluR1α immunoreactivities. Although the detection signal of silver/gold particles is the same for the two mGluRs, their differential subcellular position, in conjunction with appropriate control experiments using each antibody alone or in combination with labelling for VIP, makes it possible to identify the location of each receptor. Along plasma membranes, immunolabelling for mGluR1α was found only at the cytoplasmic face of dendritic

and somatic membranes, and at synapses it was enriched in a perisynaptic position at the edge of the postsynaptic density (Baude *et al.*, 1993; Lujan *et al.*, 1996, 1997). No presynaptic labelling was detected in sections reacted only for mGluR1α. In contrast, immunolabelling for mGluR7a was found in the presynaptic active zone (Fig. 5). Therefore, using serial sections, the vast majority of immunoparticles could be allocated to pre-(mGluR7a) or postsynaptic (mGluR1α) elements (Fig. 5). When the plasma membranes are cut tangentially, it is not possible to establish on which of the two apposed membranes the particles are located, but from other segments of the membrane, as well as from images obtained following tilting the sections, the majority of profiles can easily be characterized for the location of labelling.

The sample of postsynaptic dendrites analysed was taken as above for VIP positive terminals. Out of 24 dendritic profiles receiving at least one of each of VIP and mGluR7a positive axon terminals, 10 (42%) received at least one double-labelled (VIP and mGluR7a) axon terminal as well as other mGluR7a labelled boutons. Another 11 dendritic profiles (46%) received double-labelled axon terminals, but on the examined segment no other mGluR7a positive terminal. Only three (13%) dendritic profiles received synapses from VIP positive axon terminals which were immunonegative for mGluR7a, but synapses from other boutons forming type I synapses on the same dendritic profiles were mGluR7a positive. Regarding our selected dendritic sample, the targets of mGluR7a and VIP positive terminals, all but three (12.5%) dendritic profiles in the above sample were immuno-
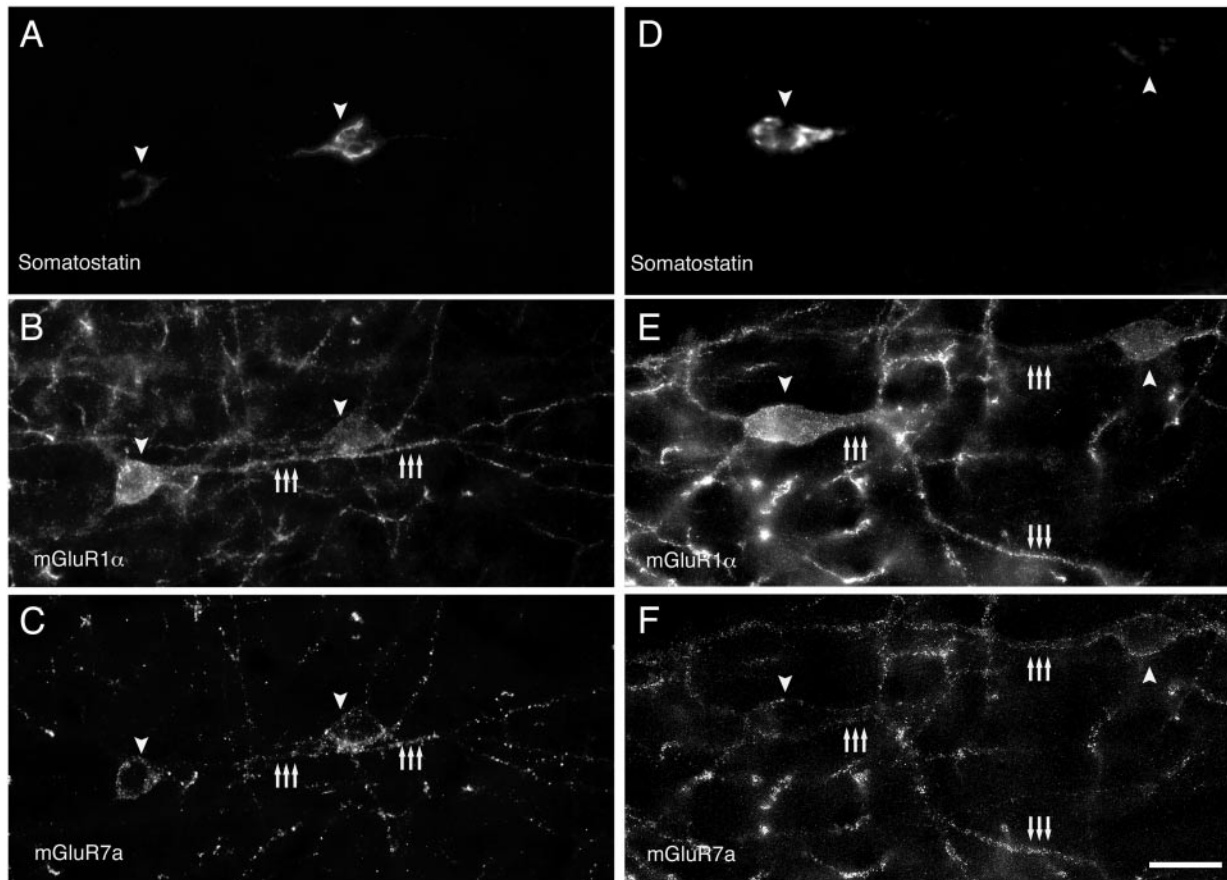
Figure 4. Most somatostatin immunopositive neurons express high levels of mGluR1α in the isocortex of adult (*A–C*) and young (P17, *D–F*) rats and are targets of mGluR7a-expressing terminals. Triple immunofluorescence for somatostatin (*A, D*), mGluR1α (*B, E*) and mGluR7a (*C, F*) shows that dendrites (arrows) originating from somatostatin neurons (arrowheads) are decorated by terminals expressing a high level of mGluR7a (arrows). Virtually all of the mGluR1α positive profiles are decorated by mGluR7a positive terminals. Scale bar: 20μm.

positive for mGluR1α. The three dendritic profiles that were unlabelled for mGluR1α were the targets of VIP and mGluR7a double-labelled terminals. None of the postsynaptic dendrites in this sample was VIP positive. There was no statistically significant difference in the frequencies of dendritic profiles targeted by terminals labelled for a cell type marker (VIP or GAD) and mGluR7a between the three types of experiments (GAD and mGluR7a; VIP and mGluR7a; VIP and mGluR7a and mGluR1α) reported above (χ^2 = 3.89, *P* > 0.1).

Parvalbumin Immunopositive Neurons and mGluR7a Immunoreactivity

Several classes of cortical GABAergic neuron express parvalbumin. The two major populations are the basket cells innervating the soma and proximal dendrites (Hendry *et al.*, 1989; Kawaguchi and Kubota, 1993) and axo-axonic cells innervating pyramidal axon initial segments (De Felipe *et al.*, 1989; Kawaguchi and Kubota, 1998). Because parvalbumin-expressing cortical neurons also innervate other interneurons, we tested whether these terminals express mGluR7a. Very few parvalbumin immunopositive axon terminals were found to give synapses to dendritic shaft profiles innervated by mGluR7a positive terminals. This was not a result of poor immunoreactivity for parvalbumin, because immunopositive terminals were of the expected frequency on various parts of pyramidal cells and other interneurons, which themselves were strongly labelled for parvalbumin. No mGluR7a immuno-

reactivity was found in parvalbumin positive terminals innervating pyramidal cell somata or other parvalbumin positive cells. In a sample of 15 parvalbumin positive boutons encountered as making synapses on mGluR7a-decorated dendrites, none was labelled for mGluR7a (Fig. 6).

Strongly parvalbumin immunoreactive somata and dendrites received mostly mGluR7a negative synaptic input. The mGluR7a immunopositive terminals innervating strongly parvalbumin positive cells were very weakly labelled. Some weakly parvalbumin immunoperoxidase positive dendrites, however, received strongly mGluR7a immunopositive innervation as revealed by immunogold labelling. Some or all of these dendrites may originate from interneurons analysed quantitatively above, which may express parvalbumin at a lower level than the majority of parvalbumin positive cells.

Quantitative Distribution of GABAergic and mGluR7a-expressing Boutons on Dendritic Profiles

The relative frequency of boutons giving synapses to dendrites receiving synapses from at least one putative GABAergic and one mGluR7a labelled terminal is shown in Figure 7. These selection criteria increased the relative proportion of GABAergic terminals in the sample; therefore, their frequency is not representative of the proportion of GABAergic input to these postsynaptic neurons. Because of the overall small proportion of GABAergic boutons on interneuron dendrites, it would have been impossible to obtain a representative sample of GABAergic
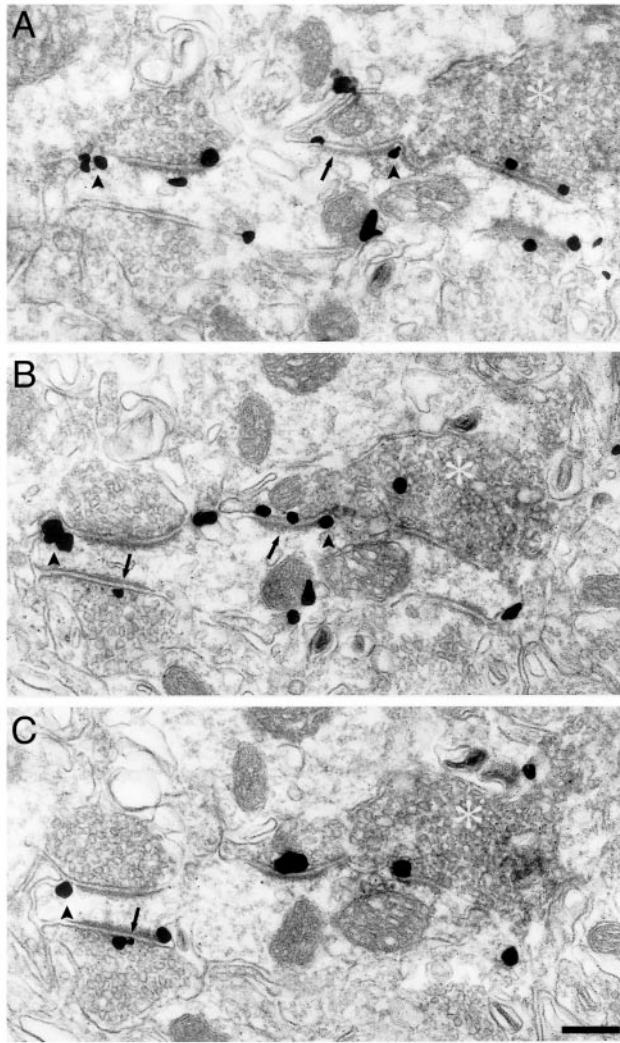
Figure 5. Electron micrographs of triple immunolabelled serial sections showing a VIP immunopositive terminal (peroxidase labelling, asterisk) making a type II synapse on a dendritic profile expressing peri- and extrasynaptic mGluR1α (e.g. arrowheads). The VIP terminal is immunopositive for mGluR7a (gold/silver particles) within the presynaptic active zone. Other mGluR7a positive terminals make type I synapses on the same dendrite (arrows) and are immunonegative for VIP. Most of the gold/silver particles can be allocated to representing either mGluR1α or mGluR7a immunoreactivity, because they are attached to the intracellular face of the plasma membrane of the dendrite or the terminals, respectively. Scale bar: 0.2 μm.

Figure 6. Parvalbumin immunopositive terminals rarely make synapses on isocortical mGluR7a-decorated dendrites and usually are immunonegative for mGluR7a. Electron micrograph of a parvalbumin positive terminal (peroxidase labelling) making a type II synapse (arrowhead) on a dendritic profile. The parvalbumin positive terminal is negative for mGluR7a, although two terminals next to it are immunopositive for mGluR7a (arrows). The terminal on the left makes a type I synapse. The immuno-particles in the dendrite may represent an intracellular pool of the receptor or have resulted from an unusual background labelling. Scale bar: 0.2 μm.

boutons from random samples of mGluR7a-labelled terminals. In our sample, the GABAergic, mGluR7a-expressing terminals constitute a considerable proportion (~38%) of all boutons. The proportion of mGluR7a-labelled terminals, which made type I synapses (mostly glutamatergic), is slightly more than that of the double-labelled terminals (~48%). The ratio of double-labelled GABAergic terminals to putative glutamatergic terminals is 0.93 ± 0.17 (mean ± SEM, range 0.66–1.25). Accordingly, in our sample of dendritic profiles, the mean probability for an mGluR7a-expressing terminal to be GABAergic is 0.48 (i.e. 0.93/1.93).

Some of the dendritic profiles received a synapse from a double-labelled bouton without receiving a synapse from another mGluR7a positive bouton within the analysed series of sections. This result may be interpreted in two ways: (i) there is a subpopulation of dendr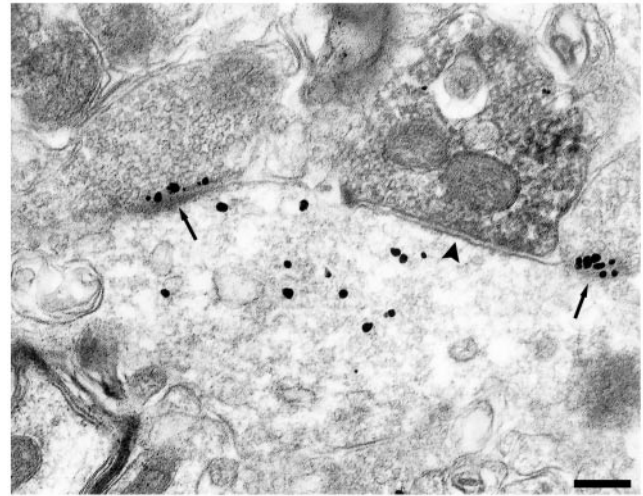ites that receive boutons amongst which only GABAergic boutons are mGluR7a labelled; (ii) the cutting plane of these profiles, together with the low number of serial sections examined, excluded the detection of other boutons. To examine these possibilities, we tested the correlation between the number of synapses encountered on a profile and the different categories of synapses (Fig. 8). The number of boutons labelled only for mGluR7a was an almost perfect linear function of the total number of boutons that were found to make synapses with a given dendritic profile (Fig. 8*C*, data pooled from all the experiments). Moreover, the slope (0.93 mGluR7a positive boutons/all boutons) and the intercept (–1.47 mGluR7a positive boutons) showed that under our conditions of labelling and searching criteria, it was rather improbable to find a bouton that was only mGluR7a positive, when the dendritic profile received less than three synapses in total. In contrast, the detection of boutons of the other types (Fig. 8*A*,*B*,*D*) was not statistically dependent on the total number of boutons examined, and we did not find significant correlations [*P* > 0.05 (Zar, 1999)]. These calculations predict that the subset of dendrites, which received only double positive boutons in the examined section series, were cut most likely in such a way that too few synapses appeared in the sections. This prediction is supported by the finding that in all the experiments, on the 40 dendritic profiles receiving mGluR7a positive synapses only from a double-labelled terminal, there were on average only 1.6 synapses (range 1–4). Therefore, in any subsequent analysis, we do not distinguish two different populations of postsynaptic targets.

The majority of GABAergic terminals (80%), as identified by immunolabelling for GAD, expressed mGluR7a in their presynaptic active zone when they targeted mGluR7a-decorated dendrites (Fig. 9*A*). Terminals labelled for VIP form a sub-population of GABAergic boutons, and virtually all of them (98%) expressed mGluR7a when targeting mGluR7a-decorated dendrites (Fig. 9*B*). A similar proportion (87%, Fisher's exact *P* > 0.05) of those VIP positive boutons, which we directly showed as targeting mGluR1α-expressing and mGluR7a-
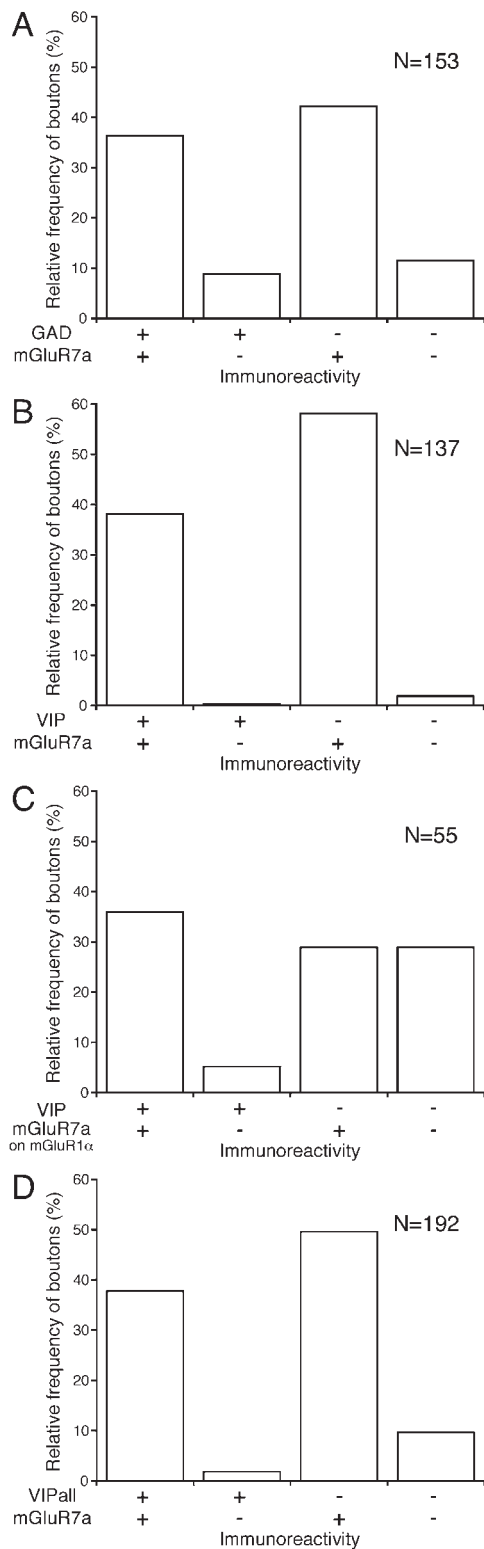
Figure 7. Relative frequency of synaptic boutons on cortical dendrites, which received at least one bouton immunopositive (+) for mGluR7a and at least one bouton immunopositive for either GAD (*A*), VIP (*B*), VIP targeting mGluR1α-expressing dendrite (*C*). The relative frequency of all VIP positive boutons shown in (*B*) and (*C*) is shown pooled in (*D*). Most of the synaptic boutons on these dendrites show immunoreactivity for mGluR7a, including the boutons originating from presumably GABAergic neurons. The selection criterion of having at least one bouton originating from a putative GABAergic neuron strongly increased their proportion and is not representative of the total population of boutons on these cells. N: number of synaptic boutons analysed.
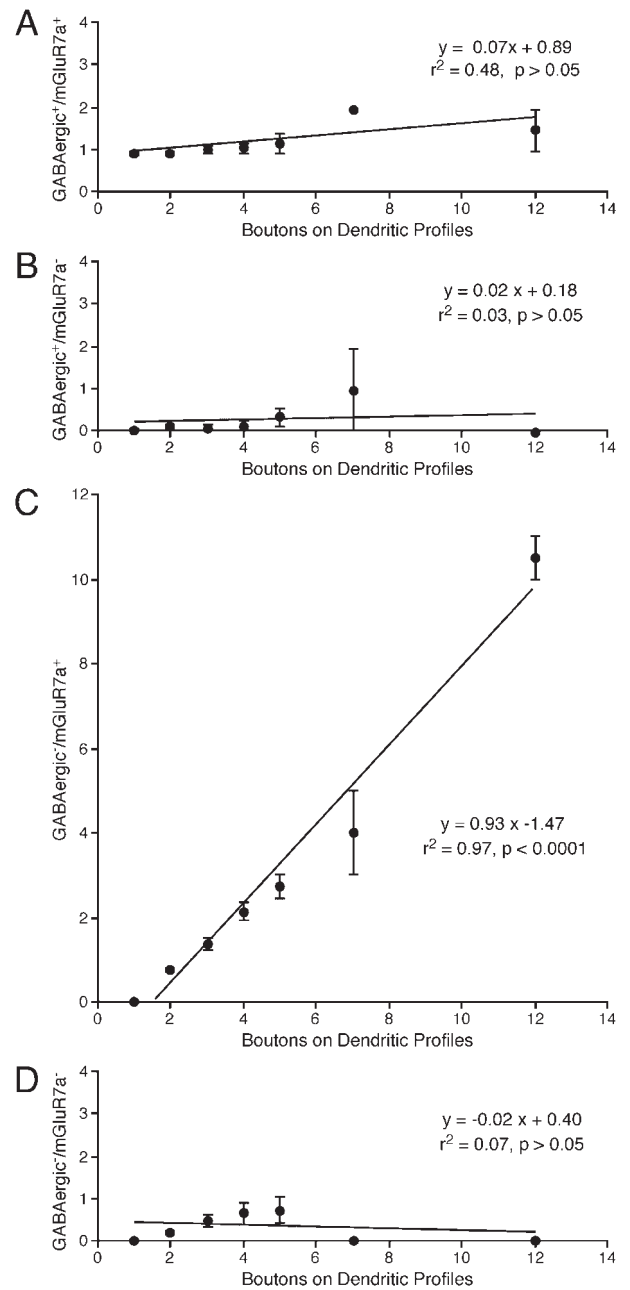

Figure 8. Dendritic shafts decorated by mGluR7a immunopositive boutons appear to form one population. The number of immunopositive and negative boutons is displayed as a function of the total number of boutons targeting an individual dendritic profile. Data are pooled from all experiments and the boutons are classified as double immunopositive for a GAD or VIP and mGluR7a (*A*), immunopositive only for GAD or VIP (*B*), immunopositive only for mGluR7a (*C*) and immunonegative (*D*). Only the number of boutons identified as single labelled for mGluR7a is a function of the number of the total synaptic boutons on each dendritic profile (*C*). This indicates that, under our selection criterion, the lack of detection of mGluR7a-labelled type I synapses on some of the dendritic profiles is due to the small number of total synapses detected on these profiles as a result of undersampling. Points represent the mean ± SEM. Lines represent the best linear fit and are defined by the equations shown. p: the probability for the slopes being equal to zero.

decorated dendrites, was also mGluR7a positive (Fig. 9*C*). Therefore, we pooled the data on VIP positive boutons. The pooled data for VIP terminals showed that 95% of them also expressed mGluR7a in their presynaptic active zone (Fig. 9*D*).
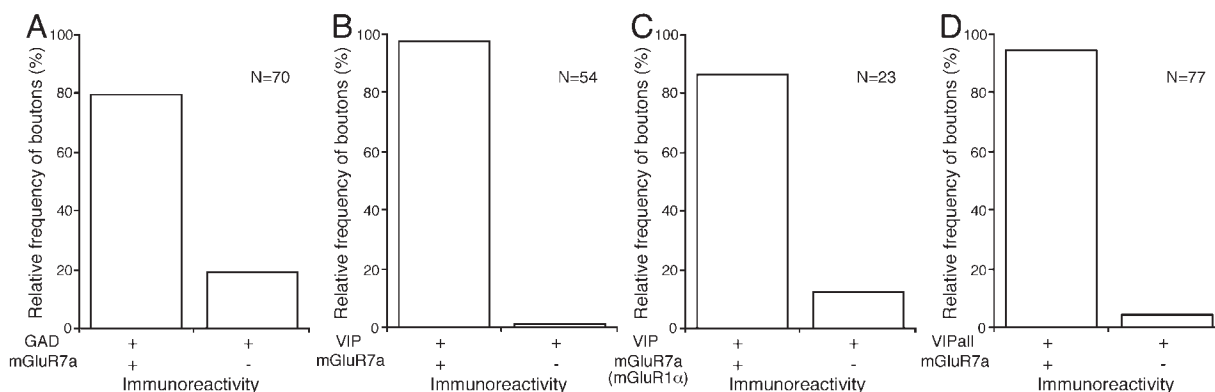
Figure 9. Proportions of mGluR7a immunopositive synaptic boutons as a fraction of the total boutons positive for a GABAergic marker, such as GAD (*A*) and VIP (*B–D*). The vast majority of GABAergic synaptic boutons express mGluR7a at their presynaptic active zone. (*B*) The frequency of double-labelled VIP-expressing boutons is shown separately from experiments in which the immunoreactivity of the target dendrite was either tested (*C*) or not tested for mGluR1α (*A*), as well as the two populations pooled (*D*). The proportion of mGluR7a immunopositive boutons in the population of the VIP terminals targeting uncharacterized dendritic targets (*D*) is higher ($P < 0.02$, χ^2 test) than the corresponding proportion in the population of GAD immunopositive boutons (*A*). N: number of synaptic boutons analysed.

This frequency is 15% higher (Fisher's exact $P < 0.02$) than that of the general GABAergic bouton population identified by labelling for GAD. These results suggest that terminals originating from VIP neurons are specifically enriched in mGluR7a and that some other GABAergic bouton population innervating the same interneuron population may not express a detectable level of mGluR7a. Future quantitative studies should address the comparison of different VIP positive terminals depending on their postsynaptic targets; this aspect of target cell specific receptor expression was beyond the scope of this study.

Discussion

We have demonstrated that the synaptic inputs to isocortical somatostatin/mGluR1α-expressing GABAergic neurons are enriched in mGluR7a located in the presynaptic active zone, outlining these cells. Synaptic terminals to dendritic spines of pyramidal cells and to the dendritic shafts of other interneurons contained a lower level of mGluR7a and were not outlined. Both the glutamatergic and the GABAergic terminals to somatostatin/mGluR1α-expressing neurons contained mGluR7a. A major source of mGluR7a-expressing GABAergic terminals is from VIP positive interneurons, which may selectively innervate the somatostatin positive cells. Virtually all VIP positive terminals innervating mGluR7a-decorated neurons expressed mGluR7a. These features of synaptic organization are homologous to those of the hippocampus (Baude *et al.*, 1993; Acsady *et al.*, 1996; Shigemoto *et al.*, 1996, 1997; Somogyi *et al.*, 1999).

Target Cells of Terminals Enriched in mGluR7a
Variability of immunolabelling between nerve terminals in pre-embedding experiments can be the result of differential receptor expression or the variable access of the antibodies to the protein in the incubated sections. The high level of mGluR7a expression around some interneuron dendrites, together with the high probability of a bouton being labelled on these dendrites, in comparison to boutons on other targets in the surrounding neuropil in both light and electron microscopic preparations, show that comparisons can be made between populations of neighbouring synaptic junctions, particularly with the help of serial sections. The use of serial sections reduced the stochastic noise arising from differences in antigen exposure. Therefore, differences in the frequency of immuno-labelling of terminals targeting different postsynaptic elements, e.g. dendritic shafts or spines, most likely reflect a true variability in receptor expression.

The level of presynaptic mGluR7a appeared to be highly variable in the presynaptic active zones. Those terminals innervating dendritic spines, most of which originate from pyramidal cells, were either immunonegative or expressed differing levels of immunoreactivity, up to a level as high as some terminals innervating mGluR7a-decorated interneuron dendritic shafts. This variability of presynaptic receptor in spine synapses predicts functional heterogeneity. A subpopulation of terminals innervating dendritic shafts contained a very high level of presynaptic receptor. As shown by our immunofluorescence results, the target cells were the somatostatin-expressing GABAergic neurons, as in the hippocampus. On these cells, the majority of terminals were enriched in mGluR7a; therefore, it is likely that the postsynaptic cell influences presynaptic receptor levels. It is known from immunocytochemical (Baude *et al.*, 1993; Stinehelfer *et al.*, 2000) and *in situ* hybridization experiments (Kerner *et al.*, 1997) that the majority (70–80%) of isocortical interneurons strongly expressing mGluR1α contain somatostatin, and most of the somatostatin positive neurons express mGluR1α. Isocortical VIP positive interneurons also express mGluR1α mRNA (Cauli *et al.*, 2000) and immuno-fluorescence studies from our laboratory show that hippocampal VIP neurons are weakly positive for mGluR1α (Ferraguti *et al.*, 2001). In this study, we could not find mGluR7a-expressing terminals targeting VIP/mGluR1α positive dendritic profiles, but this is not surprising in view of the weak dendritic labelling for VIP in our material. Other types of interneurons rarely express mGluR1α (Cauli *et al.*, 2000). Taking into account these results, it appears that the dendrites innervated by highly mGluR7a-enriched terminals mainly originate from somatostatin positive interneurons.

Somatostatin-expressing neurons are probably heterogeneous in the isocortex; therefore, further studies are required to test whether they all receive mGluR7a-enriched input. Somatostatin-expressing neurons have been classified mainly as regular spiking, and only rarely as burst firing cells (Kawaguchi and Kubota, 1996; Cauli *et al.*, 1997, 2000). Previous reports show that somatostatin neurons target mostly thin and medium size dendrites of pyramidal cells (Hendry *et al.*, 1989; Kawaguchi and Kubota, 1997).

Sources of Terminals Expressing mGluR7a

The vast majority of terminals immunopositive for mGluR7a made type I synapses and probably originated from cortical pyramidal cells. Indeed, local pyramidal cells are a main source of glutamatergic input to somatostatin-expressing cells (Reyes *et al.*, 1998; Rozov *et al.*, 2001). One source of type II synapses is from VIP positive terminals, probably of local origin. All of the cortical VIP neurons are GABAergic (Kubota *et al.*, 1994; Bayraktar *et al.*, 1997; Cauli *et al.*, 1997). In the present study, we show that most of the terminals originating from these neurons express mGluR7a when they give synapses to mGluR7a-decorated dendrites, which most likely originate from somatostatin/mGluR1α-expressing interneurons. Interneurons expressing VIP also innervate each other and pyramidal cells, but it remains to be established if the same presynaptic cell innervates several classes of cell, or if individual VIP cells are specialized to target distinct postsynaptic cell types as in the hippocampus (see below). With our experimental design it was not possible to distinguish whether the mGluR7a positive VIP terminals belong to one or more of the various electro-physiologically or neurochemically defined subpopulations of VIP-expressing neurons. Isocortical VIP neurons have been described as burst/regular or irregular spiking nonpyramidal cells (Cauli *et al.*, 1997; Kawaguchi and Kubota, 1997). If a VIP/mGluR7a-expressing cell innervates several postsynaptic cell types, it will be interesting to test if the level of presynaptic mGluR7a is expressed in a target cell specific manner, as in the case of pyramidal cell axons (Shigemoto *et al.*, 1996). In addition to presynaptic mGluR7a, the synaptic input to somatostatin-expressing cells is also under the control of presynaptic $GABA_B$ receptors, which may be activated by the dendritic release of GABA (Zilberter *et al.*, 1999; Zilberter, 2000).

The Control of GABA Release by mGluRs

Recent studies have shown that the release of GABA is under the inhibitory control of group II mGluRs in cell cultures (Schaffhauser *et al.*, 1998; Zhao *et al.*, 2001), the cerebellum (Mitchell and Silver, 2000) and the hippocampus (Poncer *et al.*, 2000). Group III mGluRs also depress GABA release in the thalamus (Salt and Eaton, 1995), the cerebellum (Mitchell and Silver, 2000), the substantia nigra pars reticulata (Wittmann *et al.*, 2001), the hypothalamus (van den Pol *et al.*, 1998), the entorhinal cortex (Woodhall *et al.*, 2001) and the hippocampus (Kogo *et al.*, 1999; Semyanov and Kullmann, 2000). Previous studies have shown that mGluR7a is expressed by GABAergic presynaptic terminals in the islands of Calleja (Kinoshita *et al.*, 1998); presynaptic terminals making type II synapses were also immunopositive in the hippocampus (Shigemoto *et al.*, 1997) and globus pallidus (Kosinski *et al.*, 1999). Recent studies indicate that glutamate, originating either from neighbouring glutamatergic synapses (Rusakov *et al.*, 1999; Mitchell and Silver, 2000; Semyanov and Kullmann, 2000), or released by the postsynaptic dendrite (Morishita *et al.*, 1998; Morishita and Alger, 1999; Zilberter, 2000), can reach mGluRs on GABAergic axons and terminals and cause depression of neurotransmission. However, glutamate has a very low efficacy on mGluR7a in heterologous expression systems (Okamoto *et al.*, 1994; Wu *et al.*, 1998); if this also applies *in situ*, it would restrict receptor activation to exceptional circumstances when glutamate concentration reaches several hundred μM in the GABAergic synaptic cleft. Alternatively, as glutamate is unlikely to be released by the GABAergic terminal, mGluR7a may be activated by high concentration of glutamate released by the dendrite, or by nearby glial processes (Pasti *et al.*, 2001). Endogenous sub-

stances other than glutamate, e.g. l-serine-*O*-phosphate, a potent group III receptor agonist, may be also released from various neuronal or glial elements. The location of mGluR7a in the presynaptic active zone is unlikely to be related to the low efficacy of glutamate as agonist, because all group III mGluRs are preferentially located in the active zone (Shigemoto *et al.*, 1997), and another receptor, mGluR8a, on which glutamate has a much higher efficacy, is co-localized with mGluR7a in the piriform cortex (Wada *et al.*, 1998) and is also concentrated in isocortical GABAergic synapses (Dalezios *et al.*, 2001).

Comparison of Receptor-defined Circuits in the Isocortex and the Hippocampus

The vast majority of isocortical GAD-labelled terminals, which make synapses with dendritic profiles decorated by mGluR7a-expressing boutons, also express mGluR7a in their presynaptic active zone, as they do in the hippocampus (Somogyi *et al.*, 1999; Dalezios *et al.*, 2001). As in the isocortex, GABAergic terminals expressing mGluR7a originate mainly from VIP positive neurons also in the hippocampus (Somogyi *et al.*, 1999; Dalezios *et al.*, 2001). Taken together, these comparisons show that isocortical GABAergic synaptic terminals targeting mGluR7a-decorated interneurons are homologous in their synaptic and molecular organization, and probably also in their function, to their counterparts in the CA1 area of the hippocampus.

In contrast to the isocortex, in the hippocampus we found that parvalbumin terminals, targeting dendritic profiles receiving mGluR7a-rich glutamatergic boutons, weakly express mGluR7a (Dalezios *et al.*, 2001). In the isocortex, we rarely found parvalbumin terminals targeting interneuron dendrites and the few parvalbumin positive terminals identified were immunonegative for mGluR7a. This is in agreement with a recent study showing that fast spiking parvalbumin isocortical interneurons rarely express mRNA for group III mGluRs (Cauli *et al.*, 2000). Isocortical parvalbumin terminals target somata, proximal dendrites and axon initial segments of pyramidal cells (Kawaguchi and Kubota, 1998) or they target, in a basket-like manner, VIP neurons (Staiger *et al.*, 1997). Moreover, a recent report on the somatosensory cortex of the rat (Gupta *et al.*, 2000) implies that large basket cells, possibly the parv-albumin-expressing variety, give synapses to Martinotti cells, known to express somatostatin (Kawaguchi and Kubota, 1996). The distribution of isocortical GABAergic terminals expressing mGluR7a is partially consistent with these reports. The lower proportion of double-labelled terminals in the GAD positive population in comparison with the VIP positive one, is explained if some parvalbumin positive terminals, which do not express mGluR7a, account for the GAD positive/mGluR7a negative terminals. As in the hippocampus (Poncer *et al.*, 2000), presynaptic group II mGluRs are probably also involved in controlling GABA release, as IPSPs are depressed by firing in fast spiking non-accommodating neurons, possibly representing parvalbumin positive cells (Kawaguchi and Kubota, 1997), through the activation of group II mGluRs (Zilberter, 2000).

Possible Consequences of Presynaptic mGluR7a Enrichment

The role of presynaptic mGluR7a is not known. The activation of group III mGluRs by exogenously applied agonists depresses synaptic transmission through presynaptic mechanisms in the isocortex (Jin and Daw, 1998). Mice that develop in the absence of mGluR7 are susceptible to sensory stimulus evoked epileptic activity, and a slightly increased excitability in hippocampal

slices (Sansig *et al.*, 2001). These observations suggest a role for mGluR7 in the frequency-dependent regulation of glutamate release. Furthermore, recordings from isocortical bitufted cells, which we show in this study to be innervated by mGluR7a-enriched synaptic input, revealed that the recovery from facilitation was much slower in the mGluR7 deficient mice (Sansig *et al.*, 2001).

Some somatostatin-expressing neurons are very sensitive to AMPA-induced excitotoxicity and this property may be connected to the abundance of GluR1 and GluR2 subunit-containing AMPA receptors and mGluR1α in their membrane (Stinehelfer *et al.*, 2000). The expression of a high level of mGluR7a may be a protection mechanism limiting glutamate release when its extracellular concentration is high. However, the presence of mGluR7a also on their GABAergic input terminals, presumably depressing GABA release when activated, would lead to disinhibition and might contribute to the excitotoxic effects of excessive AMPA receptor activation. Disinhibition produced by group III mGluR activation was demonstrated in the somatosensory cortex using an experimental protocol involving the stimulation of vibrissae (Wan and Cahusac, 1995). Depending on the type of cell recorded, disinhibition may be caused directly by depressing GABA release from the terminals innervating the recorded cell, or by depressing the excitatory drive to GABAergic neurons innervating the recorded cell. In a few cells, the iontophoretically delivered group III agonists produced an increase in inhibition. Without the identification of the recorded cells, it is difficult to compare these interesting *in vivo* findings with the present results, but the variability in the responses points to the need of clarifying the underlying circuits.

In vitro experiments in the hippocampus indicate that in some interneurons an mGluR7-like receptor may contribute to long-term depression in synaptic efficacy evoked by high frequency stimulation (Laezza *et al.*, 1999). In the isocortex, glutamatergic terminals on the somatostatin-expressing cells have a low resting transmitter release probability as compared to terminals along the same pyramidal axon innervating other cells (Reyes *et al.*, 1998). However, the inputs show a pronounced frequency facilitation, because upon repeated activation the release probability increases (Markram *et al.*, 1998; Reyes *et al.*, 1998; Rozov *et al.*, 2001). Whether this is connected to the high level of presynaptic mGluR7a demonstrated here is not clear, but the results on mGluR7-deficient mice discussed above indicate that this might be the case. The presynaptic frequency-dependent regulation of transmitter release might produce changes in excitatory and inhibitory drive leading to activation of intrinsic membrane mechanisms that govern the action potential output pattern of the postsynaptic neurons.

The enrichment of presynaptic receptors in the input of somatostatin/mGluR1α-expressing GABAergic cells probably governs the efficacy of transmitter release at different levels of extracellular glutamate concentration, which depends on the level of network activity. The activation of these cells leads to GABA release mostly to distal dendrites of pyramidal cells. In the hippocampus, the release of GABA to distal dendrites of pyramidal cells from a population of somatostatin/mGluR1α-expressing interneuron, the stratum oriens, lacunosum-moleculare cells, activates GABA$_A$ receptors (Maccaferri *et al.*, 2000). Dendritic GABA$_A$ receptor activation, in interaction with voltage-gated cationic channels, could either decrease or increase the efficacy of excitatory inputs and their propagation to the soma. The key information needed to determine which of the many plausible scenarios are physiologically relevant to the action and activation of somatostatin/mGluR1α-expressing

GABAergic cells is the pattern of their activity *in vivo* under physiological conditions.

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