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DISTRIBUTION AND SYNAPTIC LOCALISATION OF THE METABOTROPIC GLUTAMATE RECEPTOR 4 (mGluR4) IN THE RODENT CNS

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Abstract—Group III metabotropic glutamate receptors (mGluRs) are selectively activated by L-2-amino-4-phosphonobutyrate (L-AP4), which produces depression of synaptic transmission. The relative contribution of different group III mGluRs to the effects of L-AP4 remains to be clarified. Here, we assessed the distribution of mGluR4 in the rat and mouse brain using affinity-purified antibodies raised against its entire C-terminal domain. The antibodies reacted specifically with mGluR4 and not with other mGluRs in transfected COS 7 cells. No immunoreactivity was detected in brains of mice with gene-targeted deletion of mGluR4. Pre-embedding immunocytochemistry for light and electron microscopy showed the most intense labelling in the cerebellar cortex, basal ganglia, the sensory relay nuclei of the thalamus, and some hippocampal areas. Immunolabelling was most intense in presynaptic active zones. In the basal ganglia, both the direct and indirect striatal output pathways showed immunolabelled terminals forming mostly type II synapses on dendritic shafts. The localisation of mGluR4 on GABAergic terminals of striatal projection neurones suggests a role as a presynaptic heteroreceptor. In the cerebellar cortex and hippocampus, mGluR4 was also localised in terminals establishing type I synapses, where it probably operates as an autoreceptor. In the hippocampus, mGluR4 labelling was prominent in the dentate molecular layer and CA1–3 strata lacunosum moleculare and oriens. Somatodendritic profiles of some stratum oriens/alveus interneurones were richly decorated with mGluR4-labelled axon terminals making either type I or II synapses. This differential localisation suggests a regulation of synaptic transmission via a target cell-dependent synaptic segregation of mGluR4.

Our results demonstrate that, like other group III mGluRs, presynaptic mGluR4 is highly enriched in the active zone of boutons innervating specific classes of neurones. In addition, the question of alternatively spliced mGluR4 isoforms is discussed. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: basal ganglia, hippocampus, presynaptic, electron microscopy.

Metabotropic glutamate receptors (mGluRs) form a family of G-protein-coupled receptors that includes eight different subtypes, named mGluR1–mGluR8, which are classified into three subgroups according to their amino acid sequence homology, pharmacological profile and intracellular coupling (Pin and Duvoisin, 1995). Group I mGluRs include mGluR1 and mGluR5; group II comprises mGluR2 and mGluR3, while group III consists of mGluR4, mGluR6, mGluR7 and mGluR8.

Group III mGluRs are selectively activated by phosphono derivatives of L-glutamate, such as L-2-amino-4-phosphonobutyrate (L-AP4), and L-serine-*O*-phosphate. L-AP4 activates with low micromolar potency mGluR4, mGluR6 and mGluR8, whereas for mGluR7 concentrations higher than 100 μM are required (Schoepp et al., 1999). The activation of L-AP4-sensitive receptors generally produces depression of glutamatergic and GABAergic synaptic transmission probably by inhibiting neurotransmitter release from nerve terminals. This pre-

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Abbreviations: CA, cornu ammonis; DG, dentate gyrus; EDTA, ethylenediaminotetra-acetate; EGTA, ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; GP, globus pallidus; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid); HRP, horseradish peroxidase; IPSC, inhibitory postsynaptic current; IPTG, isopropyl-β-thiogalactopyranoside; -IR, -immunoreactivity; KO, 'knock-out' gene-targeted deletion; L-AP4, L-2-amino-4-phosphonobutyrate; MBP, maltose binding protein; mGluRs, metabotropic glutamate receptors; NGS, normal goat serum; PB, phosphate buffer; PCR, polymerase chain reaction; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; SNr, substantia nigra pars reticulata; TBS, Tris-buffered saline; VP, ventral pallidum.

synaptic inhibition, which is thought to involve calcium channel modulation (Trombley and Westbrook, 1992; Glaum and Miller, 1995; Takahashi et al., 1996), has been described in several brain areas, including the hippocampus (Koerner and Cotman, 1981; Baskys and Malenka, 1991; Gereau and Conn, 1995; Vignes et al., 1995), neocortex (Burke and Hablitz, 1994), striatum (Calabresi et al., 1993; Pisani et al., 1997), thalamus (Turner and Salt, 1999) and cerebellum (Conquet et al., 1994; Pekhletski et al., 1996). A facilitatory effect of L-AP4 on transmitter release has also been described in the entorhinal cortex (Evans et al., 2000). However, the relative contribution of the different group III mGluRs to the effects of L-AP4 in many of these brain areas remains to be clarified.

Group III mGluRs undergo alternative splicing, which appears to generate for each receptor at least two variants with different C-terminal domains (Thomsen et al., 1997; Corti et al., 1998). The alternatively spliced variants of each receptor subtype (named mGluR4a and mGluR4b, mGluR7a and mGluR7b, mGluR8a and mGluR8b) maintain unchanged the N-terminal domain and, therefore, retain the same pharmacological properties (Thomsen et al., 1997; Corti et al., 1998). To date, no distinctive functional roles for the different splice variants of group III mGluRs have been identified. However, mGluR7a and mGluR7b were shown to differ in their protein expression pattern and transcript abundance (Shigemoto et al., 1997; Kinoshita et al., 1998; Corti et al., 1998). There are no data on the localisation of the reported mGluR4b isoform either at the mRNA or protein level (Thomsen et al., 1997). The localisation of mGluR4a, hereafter referred to as mGluR4, has been investigated in several brain areas (Kinoshita et al., 1996; Shigemoto et al., 1997; Mateos et al., 1998; Bradley et al., 1999; Benitez et al., 2000; Azkue et al., 2001).

The role and localisation of mGluR4 has been well established in the cerebellum, where the application of L-AP4 reversibly depresses excitatory postsynaptic potentials (EPSPs) at parallel fibre–Purkinje cell synapses (Conquet et al., 1994; Pekhletski et al., 1996). The only group III mGluR expressed by granule cells is mGluR4 (Ohishi et al., 1995; Saugstad et al., 1997). The presence of the mGluR4 protein on parallel fibre terminals (Kinoshita et al., 1996), and specifically at the presynaptic membrane (Mateos et al., 1998), has recently been shown by immunocytochemical studies. On the other hand, in other brain areas, such as the basal ganglia and the hippocampus, data on mGluR4 are scarce or inconsistent.

In the hippocampus, L-AP4 causes reversible depression of synaptic input to principal neurones in several synaptic pathways, including the Schaffer collateral input to CA1 pyramidal cells (Baskys and Malenka, 1991; Gereau and Conn, 1995; Vignes et al., 1995), the mossy fibre input to CA3 pyramidal cells (Manzoni et al., 1995) and perforant path input to granule cells (Koerner and Cotman, 1981; O'Leary et al., 1997). Furthermore, L-AP4 mediates presynaptic inhibition of excitatory inputs to dentate hilar-border (Doherty and Dingledine, 1998) and CA1 interneurones (Scanziani et al., 1998), and depression of GABAergic transmission among CA1 interneurones (Semyanov and Kullmann, 2000). Most of these events have been suggested to involve mGluR4 due to the high efficacy of L-AP4 (Tanabe et al., 1993; Cavanni et al., 1994; Eriksen and Thomsen, 1995) and to the relatively high levels of mRNA expression in the entorhinal cortex, CA3 area and the hilus (Ohishi et al., 1995). However, immunoreactivity for mGluR4 in the hippocampus was found restricted to the neuropil of the stratum lacunosum moleculare of cornu ammonis (CA) and to the inner third of the dentate molecular layer (Shigemoto et al., 1997; Bradley et al., 1999). Labelling for mGluR4 was seen in the active zone of axon terminals forming primarily type I (asymmetrical) synapses on spines of granule cells, although it has also been reported in both type II (symmetrical) and type I synapses on dendrites (Shigemoto et al., 1997).

In the basal ganglia, the striatum expresses a high level of mGluR4 mRNA (Ohishi et al., 1995). In addition, the striatum, the globus pallidus (GP) and the substantia nigra pars reticulata (SNr) were reported to contain a high density of [^{3}H]L-AP4 binding sites (Hudtloff and Thomsen, 1998). When the binding of [^{3}H]L-AP4 was examined in brain sections of mice with gene-targeted deletion ('knock-out', KO) of mGluR4 a significant decrease in binding sites was found in the SNr (Thomsen and Hampson, 1999). However, a recent immunocytochemical study (Bradley et al., 1999) has found negligible amounts of mGluR4-immunoreactivity (-IR) in the rat SNr.

The main aim of this work was to clarify the localisation of mGluR4 in the basal ganglia and hippocampus. For this purpose, we have developed and characterised polyclonal antibodies raised against the entire carboxyl-terminal domain of the rat mGluR4 and used these to study the cellular and subcellular distribution of mGluR4 in the rat and mouse brain. The specificity of the antibodies was established in mGluR4-KO (−/−) mice and COS 7 cells expressing mGluR subtypes.

EXPERIMENTAL PROCEDURES

Production of antibodies to mGluR4

A polyclonal antiserum to mGluR4 was raised using a fusion protein between the C-terminal fragment of the rat mGluR4 (amino acid residues 834–912; Tanabe et al., 1993) and the maltose binding protein (MBP). The cDNA fragment corresponding to amino acids 834–912 was amplified by polymerase chain reaction (PCR) from rat mGluR4 cDNA and subcloned into the *Eco*RI/*Xba*I sites of the plasmid pMal-C2 (New England Biolabs, Hitchin, Herts, UK). After subcloning, the PCR fragment was subjected to nucleotide sequence analysis of both strands with a dye terminator cycle sequencing ready-reaction kit (ABI Prism Perkin Elmer, Monza, Milan, Italy). The mGluR4–MBP fusion protein was induced, by adding 0.3 mM isopropyl-β-thiogalactopyranoside (IPTG), in *Escherichia coli* and subsequently purified onto an amylose resin (New England Biolabs). One rabbit (New Zealand White) was immunised by s.c. injections (∼400 μl/injection; 10 sites) of mGluR4–MBP fusion protein (100 μg/injection) using aluminium hydroxide as adjuvant (1 mg/injection), according to standard procedures

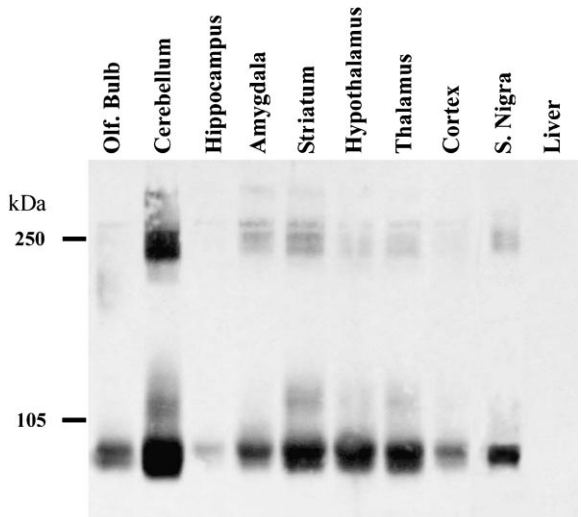

Fig. 1. Regional expression pattern of mGluR4 in the rat brain as assessed by western immunoblot with affinity-purified mGluR4 antibodies. Membrane preparations were subjected to 8% SDS–PAGE and blotted onto PVDF membranes. Immunoreactive products had an estimated molecular mass of approximately 100 and 200 kDa, consistent with the deduced molecular weight of mGluR4 and of its dimer. Liver protein extracts were used as negative control. Positions of molecular mass markers in kDa are indicated on the left.

(Harlow and Lane, 1988). After four boosts, the rabbit was exsanguinated under non-recovery anaesthesia (pentobarbitone 30 mg/kg, i.v.). To affinity purify the antibodies, a second fusion protein, mGluR4–His_6, was produced by subcloning the same PCR fragment (amino acids 834–912) into the *Sma*I site of the plasmid pET-20b (Novagen, Milan, Italy). The mGluR4–His_6 fusion protein was induced, by adding IPTG (1 mM), in *E. coli* (BL21) and purified under native conditions according to the manufacturer's instructions (Qiagen, Milan, Italy). The mGluR4–His_6 fusion protein was then coupled to CNBr-activated Sepharose 4B (Amersham-Pharmacia Biotech, Amersham, Buckinghamshire, UK) and subsequent antibody purification performed as previously described (Conquet et al., 1994).

Expression of mGluRs by transient transfection of COS 7 cells

cDNAs encoding rat mGluR1a, mGluR2, mGluR3, mGluR4, mGluR5a, mGluR5b, mGluR7a, mGluR7b, mGluR8a and mGluR8b, were subcloned into the eukaryotic expression vector pcDNA3 (InVitrogen, Groningen, The Netherlands). COS 7 cells were plated at a density of 5×10^5 cells/10 cm Petri dish and were grown in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) in the presence of 10% foetal bovine serum. Transfections were performed with a solution containing 8 μg of cDNA in 80 μl lipofectamine reagent according to the manufacturer's instructions (Life Technologies). After 48 h, cell monolayers were lysed in the culture dish by incubation with 500 μl of lysis buffer (50 mM HEPES, pH 7.4, 1 mM EGTA, 0.2% Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin) for 5 min. All chemicals were purchased from Sigma, Milan, Italy.

Immunoblotting of cell and brain membranes

Several brain areas from rat (two adult Sprague–Dawley) and mouse (two adult mGluR4$^{+/+}$ and two adult mGluR4$^{-/-}$ kindly provided by Dr D. Hampson, University of Toronto) were dissected under a stereomicroscope and membranes were prepared according to a previously published protocol (Romano et al., 1995). In brief, brain and liver tissues were homogenised with a potter at maximal speed in the following buffer: 2 mM EDTA, 0.32 M sucrose, 50 μM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml chymostatin, 1 μg/ml antipain, 1 μg/ml bacitracin, 1 μg/ml bestatin, 1 μg/ml pepstatin A. Samples were then centrifuged at $1000\times g$ for 10 min and the supernatant centrifuged at $30\,000\times g$ for 20 min. The pellet was resuspended in lysis buffer (2 mM HEPES, 2 mM EDTA in the presence of protease inhibitors) and kept on ice for 30 min. After a further centrifugation at $30\,000\times g$ for 20 min, membranes were resuspended in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl) containing protease inhibitors. Protein concentrations were determined by the bicinchoninic/BCA protein assay ac-

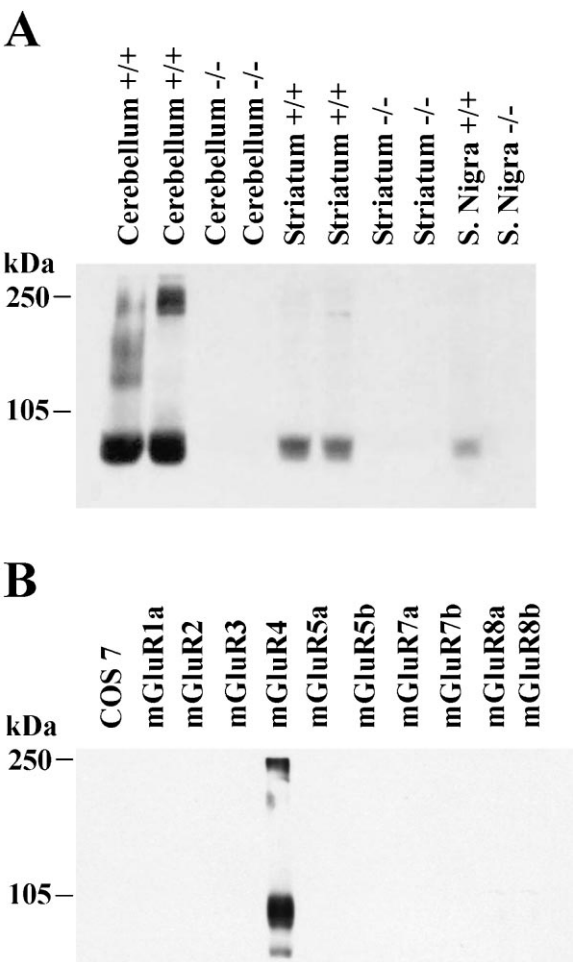

Fig. 2. Molecular specificity of the affinity-purified mGluR4 antibodies. (A) Immunoblot analysis of membrane preparations from cerebellum, striatum and substantia nigrae of two homozygous control ($^{+/+}$) and two mGluR4-KO ($^{-/-}$) mice. The substantiae nigra from the two mice in each group were pooled, whereas the striatum and cerebellum of each animal were processed separately. Membrane preparations were subjected to 8% SDS–PAGE and blotted onto PVDF membranes. Blots were reacted with the affinity-purified mGluR4 antibodies. The immunoreactive products observed in wild type mice were completely absent from membranes of mGluR4-KO mice. (B) Immunoblot analysis of COS 7 cells transiently transfected with rat cDNAs encoding: mGluR1a, mGluR2, mGluR3, mGluR4, mGluR5a, mGluR5b, mGluR7a, mGluR7b, mGluR8a and mGluR8b. Crude cellular extracts were subjected to 8% SDS–PAGE, blotted onto PVDF membranes and reacted with the affinity-purified mGluR4 antibodies. Estimated molecular masses of immunoreactive products were approximately 100 and 200 kDa, both of which were observed exclusively in the lane corresponding to the mGluR4-transfected cells. Positions of molecular mass markers in kDa are indicated on the left.

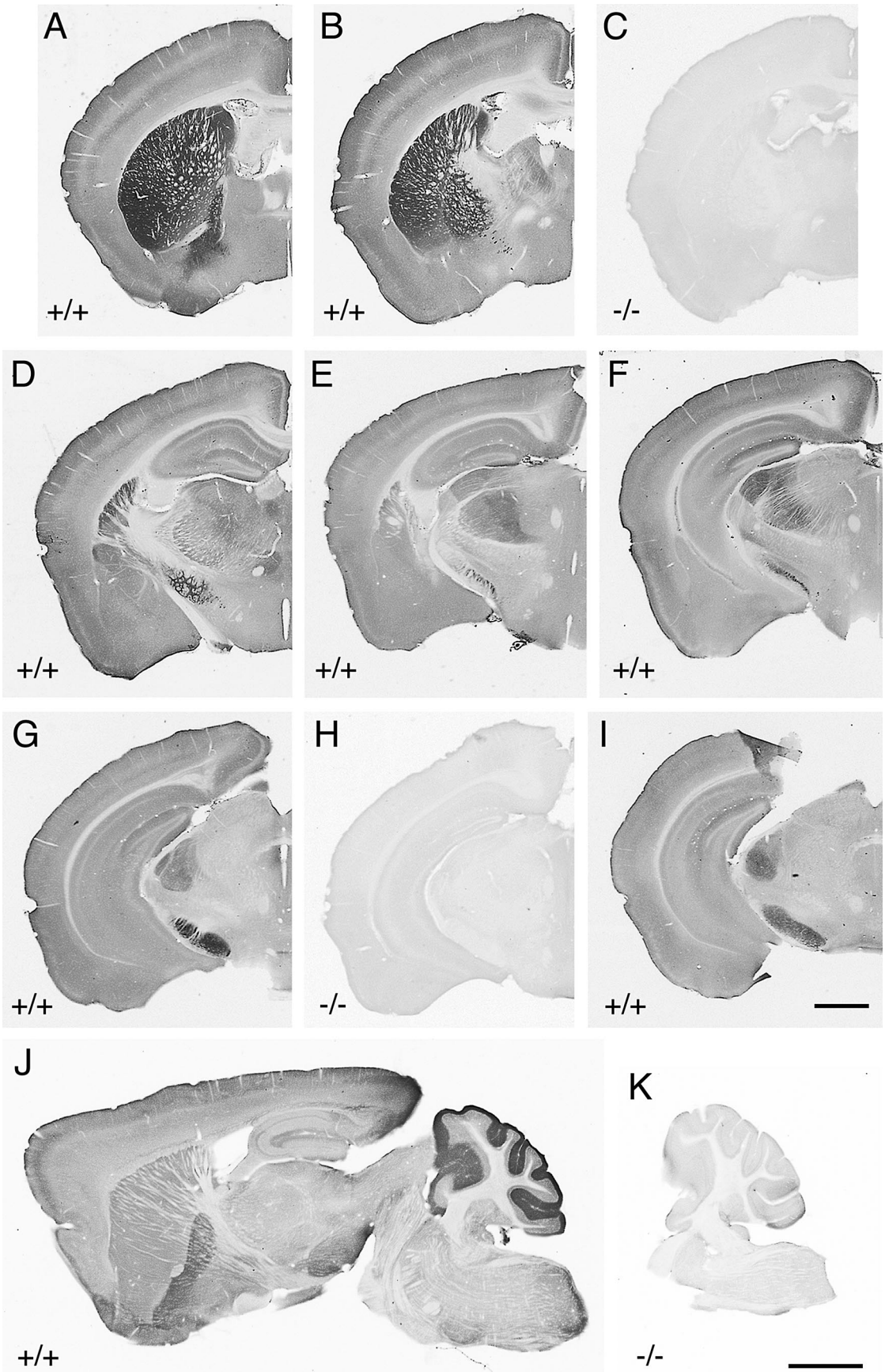

Fig. 3.

cording to the manufacturer's instructions (Pierce Chemicals, Rockford, IL, USA). Total proteins of cell lysate (10 μg/lane) and brain membranes (70 μg/lane) were denatured in Laemmli sample buffer containing 20 mM dithiothreitol and heated for 3 min at 60°C for sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on an 8% resolving gel. Proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, Hybond P, Amersham-Pharmacia Biotech). Blots were blocked for 1 h in 5% dried skimmed milk in TBS–0.1% Tween 20 and incubated overnight at 4°C with mGluR4 antibodies. Immunodetection was carried out with secondary antibodies (1:2000 anti-rabbit IgG from donkey, NA934, Amersham) conjugated to horseradish peroxidase (HRP) and by enhanced chemiluminescence (ECL Amersham).

Preparation of animals and tissue for immunocytochemistry

The experiments with animals described in this study were all carried out in accordance with the UK Animals (Scientific Procedure) Act 1986 and associated procedures. Six adult Sprague–Dawley rats (300–350 g) were deeply anaesthetised with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.1 M phosphate-buffered 0.9% saline, followed for 20 min by a fixative composed of 4% paraformaldehyde, 0.05% glutaraldehyde, ~0.2% picric acid made up in 0.1 M phosphate buffer (PB, pH 7.2). Brains were quickly removed, extensively rinsed in PB and sectioned in the coronal or sagittal plane at 50–70 μm thickness on a vibratome. Fixed (4% paraformaldehyde) mGluR4$^{+/+}$ and mGluR4$^{-/-}$ mouse brains were kindly provided by Dr D. Hampson (University of Toronto).

Pre-embedding immunocytochemistry and electron microscopy

Immunocytochemical procedures were as described earlier with minor modifications (Baude et al., 1993). In brief, sections were pre-incubated (1 h) with 20% normal goat serum (NGS) in 0.1 M PB, and subsequently incubated (72 h, 4°C) with the primary antibodies in 1% NGS–PB. Triton X-100 (0.3%) was added when the sections were prepared only for light microscopy. Immunoperoxidase reactions were carried out using the avidin–biotin–HRP complex method (ABC, Elite kit, Vector Laboratories, Burlingame, CA, USA). Pre-embedding immunogold reactions were performed using anti-rabbit IgG coupled to 1.4-nm gold particles (1:100, Nanogold, Nanoprobes, Yaphank, NY, USA). Gold particles were enhanced by silver amplification for 10–15 min using the HQ Silver kit following the manufacturer's instructions (Nanoprobes). Sections were then routinely processed for either light or electron microscopic examination. For electron microscopy, after osmium treatment (2%) and contrasting with uranyl acetate (1%), the sections were embedded in Durcupan ACM (Fluka, Gillingham, Dorset, UK). Ultrathin sections (70 nm) were collected on pioloform-coated copper slot grids. Lead citrate contrasting was used only for immunogold/silver-labelled sections.

RESULTS

Western blot analysis of mGluR4-IR in tissue and cell membranes

Polyclonal antibodies directed against the C-terminal domain of the rat mGluR4 were raised to a bacterial fusion protein between the last 79 amino acids of mGluR4 and the MBP. The antibodies were then affinity-purified using a second fusion protein, (834–912) mGluR4–His$_6$, coupled to Sepharose 4B.

Western blot analysis, using the affinity-purified polyclonal antibodies, was then performed on membrane preparations from several regions of the rat brain. Membranes extracted from liver were also used as control. Each lane was loaded with 70 μg of total protein allowing a direct quantification of mGluR4 abundance in the different regions analysed. The mGluR4 antibodies reacted with two distinct bands, one with the apparent size of 100 kDa, similar to the predicted molecular weight of the mGluR4 protein, and a second band of approximately 200 kDa, which is likely to represent receptor dimers or aggregates (Fig. 1). All brain areas analysed showed mGluR4-IR bands, although they displayed different degrees of intensity (Fig. 1). The strongest signal was observed in cerebellar membranes. Intense mGluR4-IR was present in striatum, substantia nigra, thalamus and hypothalamus, whereas intermediate immunoreactivity was observed in olfactory bulb, amygdala and neocortex. In hippocampal membranes the antibodies gave rise only to a weak signal. No bands were detected in liver protein extracts (Fig. 1). Pre-adsorption of the antibodies, using an excess of mGluR4–His$_6$ fusion protein, resulted in the complete abolition of mGluR4-IR in rat brain extracts (data not shown).

To assess the specificity of the mGluR4 antibodies, membranes prepared from cerebellum, striatum and substantia nigra of two mice lacking mGluR4 (Pekhletski et al., 1996) were analysed. Immunoblot analysis showed no reactive bands in membranes of mGluR4-KO mice (Fig. 2A). On the other hand, the expression pattern observed in brain extracts from control mice was consistent with that in rat brain. Western blot analysis was also performed on membranes of COS 7 cells transiently expressing mGluR1a, mGluR2, mGluR3, mGluR4, mGluR5a, mGluR5b, mGluR7a, mGluR7b, mGluR8a and mGluR8b, in order to evaluate the specificity of the mGluR4 antibodies relative to the other mGluR subtypes (Fig. 2B). The affinity-purified antibodies reacted only with homogenates of mGluR4 COS 7 cells, detecting two bands of approximately 100 and 200 kDa, as observed in rat and mouse tissues.

Distribution of the immunoreactivity for mGluR4 in the rat and mouse brain

The regional distribution of mGluR4 receptors was investigated by light microscopic immunocytochemistry in both rat and mouse brain. No detectable differences

Fig. 3. Distribution of the immunoreactivity for mGluR4 in the brain of homozygous control ($^{+/+}$) and mGluR4-KO ($^{-/-}$) mice. Coronal (A–I) and sagittal (J, K) sections were reacted with antibodies against mGluR4; marked immunoreactivity is seen in the striatum (A, B, J), GP (B, J), VP (A, J), entopeduncular nucleus (D–F), SNr (G, I) and cerebellar cortex (J) of control mice (A, B, D–G, I, J), whereas sections from mGluR4-KO (C, H, K) do not show any immunoreactivity. The mouse brain cut sagittally showed somewhat weaker immunoreactivity, probably due to different effectiveness of the fixation, as also observed in rat brain tissue. As a result, the difference in immunoreactivity between striatum and GP appears more evident in the sagittal section. Scale bars = 1 mm (for coronal sections A–I); 1.5 mm (sagittal sections J and K).

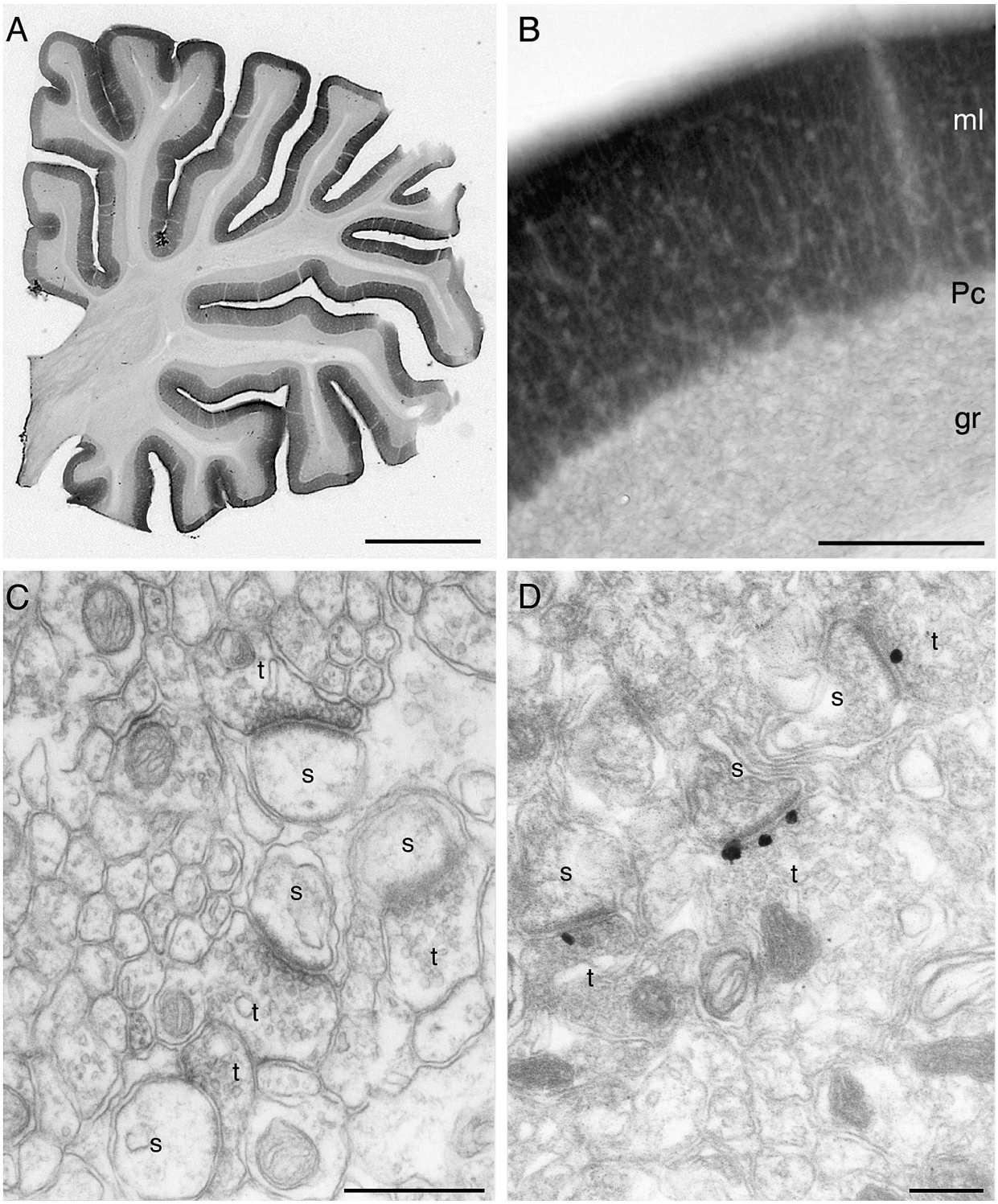

Fig. 4. Immunoreactivity for mGluR4 in the rat cerebellum. Light micrographs of (A) a parasagittal section through the vermis of the cerebellum and of (B) the molecular layer (ml) of the cerebellar cortex showing mGluR4-IR. Electron micrographs of immunoperoxidase (C) and immunogold (D) labelling for mGluR4 in the presynaptic active zone of parallel fibre axon terminals (t) making type I synaptic contacts with dendritic spines (s) in the molecular layer. Abbreviations: gr, granule cell layer; Pc, Purkinje cell layer. Scale bars = 2 mm (A); 100 μm (B); 0.5 μm (C); 0.2 μm (D).

were observed between the two species; therefore, the results described below apply to both of them. Immunolabelling with the mGluR4 antibodies showed a widespread distribution amongst areas of the brain, but discrete cellular and subcellular localisation within each area. The cerebellar cortex, GP and ventral pallidum (VP) were most intensely labelled. Robust staining was also evident in the olfactory tubercle, striatum, entopeduncular nucleus, the sensory relay nuclei of the thalamus including the medial and dorsolateral geniculate

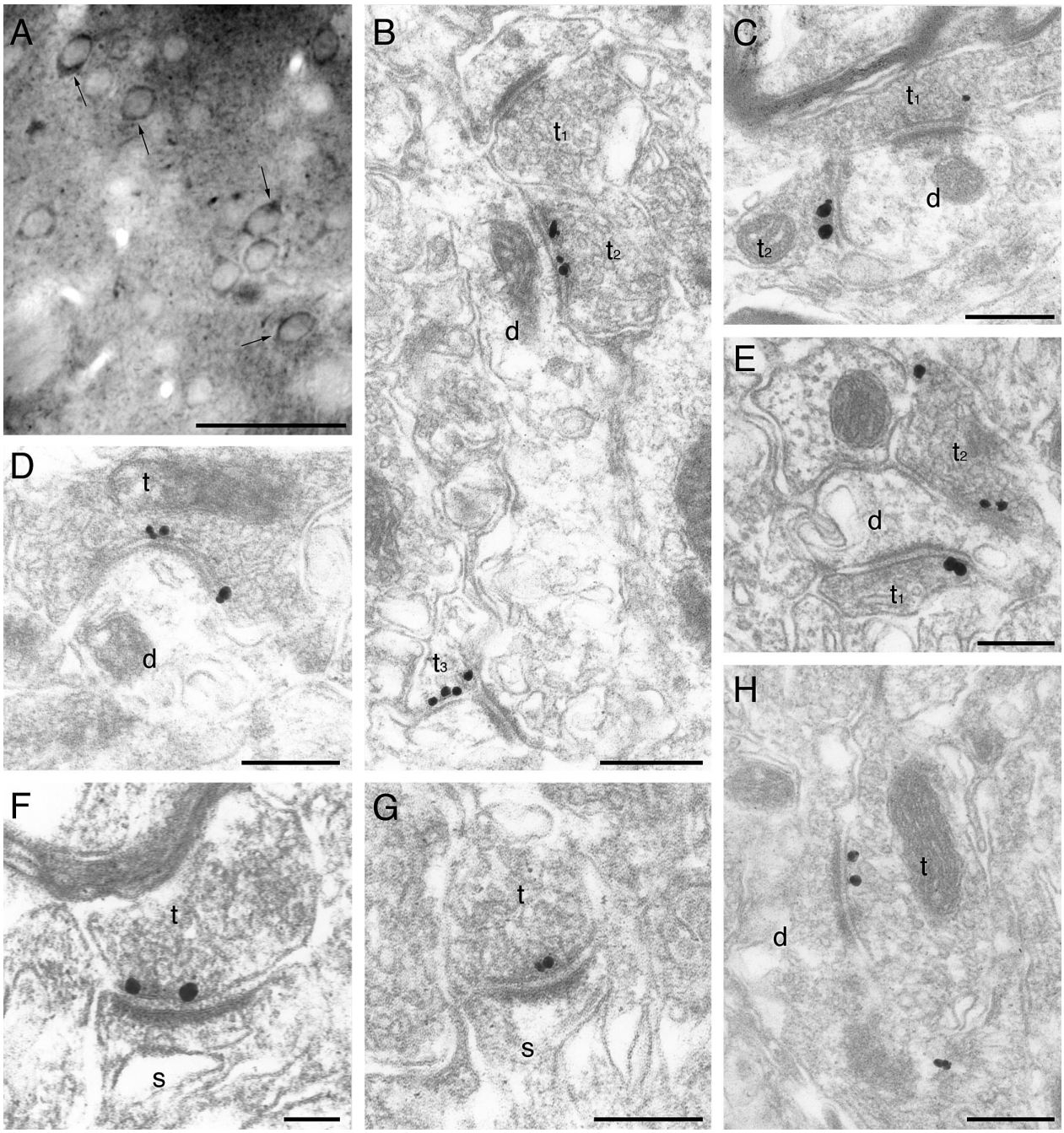

Fig. 5. Immunolocalisation of mGluR4 in the rat striatum. (A) Light micrograph of mGluR4-IR in a coronal section of the dorsal striatum. Several somata of medium spiny neurones (some indicated by arrows) show immunostaining of the cytoplasm, and numerous labelled puncta are present in the neuropil. Immunogold particles for mGluR4 concentrate in the presynaptic membrane specialisation of type II synapses on dendritic shafts (B–E) and of type I synapses on dendrites (E–H) and spines (F, G). Two type II synapses with labelled boutons (t_2 and t_3) and one unlabelled terminal (t_1) forming a type I synapse on a spine are shown in B. As depicted in E, occasional dendrites targeted by labelled terminals forming either type I (t_1) or type II (t_2) synapses are observed. Abbreviations: d, dendrite; s, spine; t, axon terminal. Scale bars = 50 μm (A); 0.3 μm (B); 0.2 μm (C–E, G, H); 0.1 μm (F).

nuclei, the substantia nigra and spinal trigeminal nucleus (Fig. 3). In the thalamus, the expression pattern of mGluR4-IR varied from intense in the posterior thalamic nuclei, the submedius and dorsolateral geniculate nucleus, to moderate or low in the anteromedial, ventral posterior and mediodorsal thalamic nuclei. Moderate to weak labelling was observed in layers I–III and V of the neocortex, in the piriform cortex, hippocampus, lateral and basolateral amygdaloid nuclei and in the superficial grey of the superior colliculus. When the antibodies were applied to brain sections of mice lacking mGluR4 expression no labelling could be detected (Fig. 3C, H, K).

In the cerebellar cortex, intense mGluR4-IR was observed in the neuropil of the molecular layer

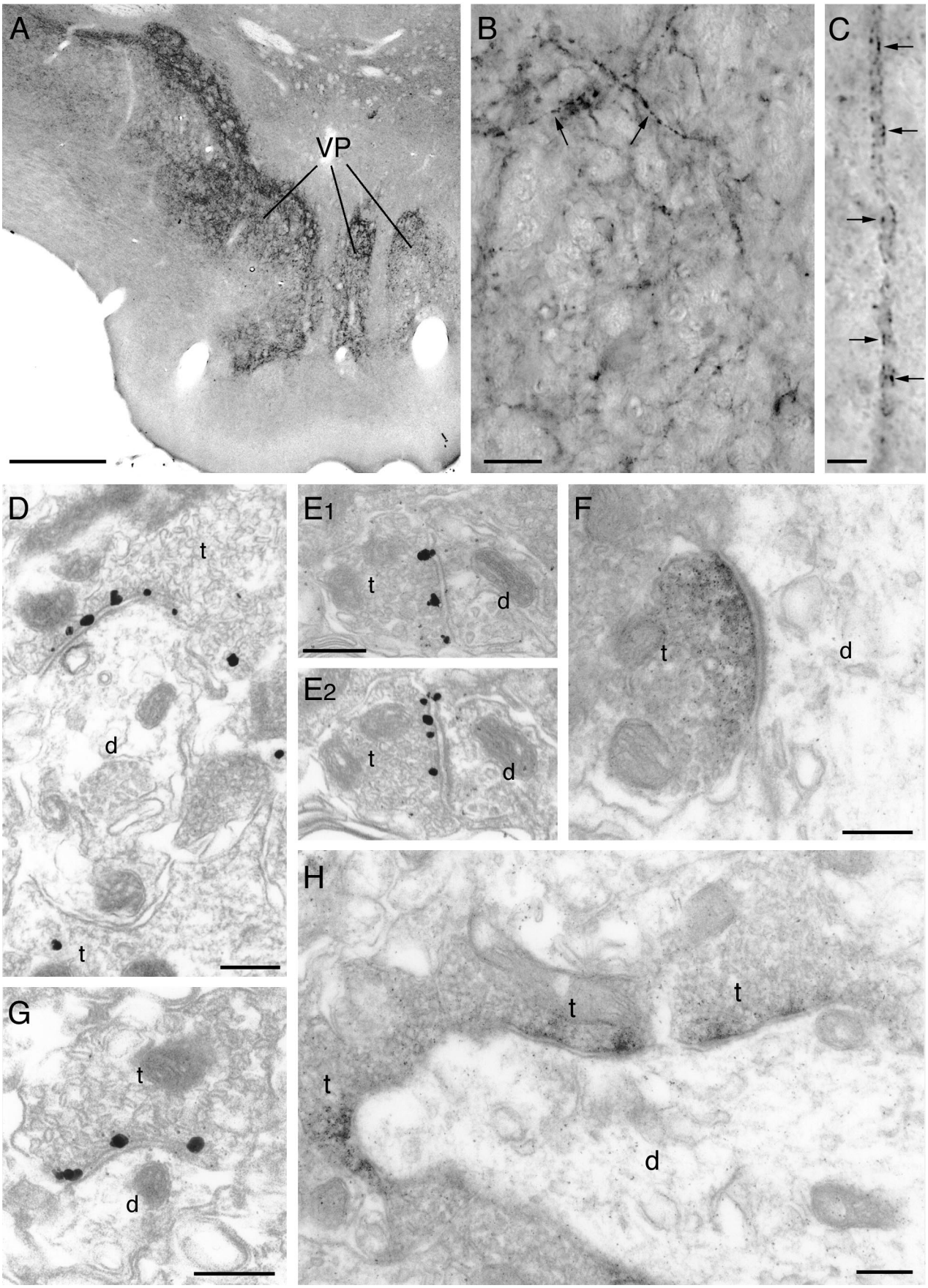

Fig. 6.

(Fig. 4A, B). Electron microscopic analysis of the rat cerebellar cortex revealed many labelled axon terminals in the molecular layer forming type I synaptic contacts with dendritic spines (Fig. 4C, D) and occasionally with non-spiny dendritic profiles. From the fine structural characteristics and frequency of the immunopositive terminals it is concluded that they are parallel fibre terminals confirming earlier studies (Kinoshita et al., 1996; Mateos et al., 1998). The peroxidase reaction product and the immunogold particles were predominantly concentrated in the presynaptic membrane specialisation (Fig. 4C, D).

Overall, mGluR4-IR was found enriched in both the dorsal and ventral division of the basal ganglia. In the striatum, mGluR4-IR consisted primarily of numerous finely stained puncta. These were present throughout the neuropil of the striatum. Larger labelled granules were also observed outlining dendrites. Some staining could be found within somata of medium spiny neurones (Fig. 5A). Electron microscopy performed on the rat dorsal striatum showed mGluR4-IR axon terminals making type II synapses with dendritic profiles (Fig. 5B–E). A few type I synapses on dendritic shafts (Fig. 5H) and spines (Fig. 5F, G) were also found labelled for mGluR4. The subcellular location of the reaction products was the same as in the cerebellar cortex.

The staining pattern observed with the mGluR4 antibodies in both the GP and VP was different from that in the striatum (Fig. 6A). Dense granular immunostaining decorated dendritic and more rarely perikaryal profiles (Fig. 6B). Most of the granules were seen in linear arrays, often demarcating dendrites on both sides (Fig. 6C). When mGluR4-IR was associated with somata, the labelling was not found in the cytoplasm but rather outlined the outer surface of neurones. Analysis at the electron microscopic level of mGluR4-IR in these structures showed immunoparticles on axon terminals forming type II synapses with dendrites (Fig. 6D–H). Similarly to the GP and VP, the mGluR4 immunolabelling in the entopeduncular nucleus and SNr consisted primarily of a dense granular pattern distributed in the neuropil, which was often associated with dendritic profiles or cell perikarya (Fig. $7A_{1-3}$). Electron microscopic analysis of the rat SNr showed labelled axon terminals that formed primarily type II (Fig. 7C–F) as well as type I synapses with dendritic shafts (Fig. 7G). Presynaptic terminals immunopositive for mGluR4 on somata established type II synapses (Fig. 7B). Immunolabelled presynaptic boutons making type II synapses often formed invaginations embracing dendritic digitations, a feature indicative of striatal terminals (Fig. 7E). The subthalamic nucleus and substantia nigra pars compacta did not show detectable mGluR4-IR.

Light microscopic examination of the hippocampus showed moderate mGluR4-IR in the neuropil of the molecular layer of the dentate gyrus (DG) and stratum lacunosum moleculare of CA areas. In addition, in CA1–CA3 stratum oriens/alveus a network of somatodendritic profiles of interneurones was decorated with intensely labelled axon terminals (Fig. 8A, B). Electron microscopic examination of the rat CA1 area showed labelled axon terminals forming both type I (Fig. 8C–E) and type II (Fig. 8F) synapses with dendritic profiles of interneurones of the stratum oriens/alveus. In this layer, labelled terminals on spines were also occasionally observed (Fig. 8G). In the pyramidal cell layer, a few type II synapses on somata of probable interneurones exhibited mGluR4-IR in the presynaptic bouton (Fig. 9A, B). In stratum radiatum scattered immunolabelled terminals on spines were found (Fig. 9C), whereas in stratum lacunosum moleculare numerous labelled terminals on spines and dendritic shafts could be detected (Fig. 9D, E).

Immunostaining in the dentate molecular layer was stronger in the inner third and weak in the middle and outer thirds (Fig. 10A). Some interneuronal dendritic profiles were decorated with strongly labelled puncta in both the DG molecular layer and the hilus (Fig. 10D). These dendrites appeared to originate from interneurones located in both the granule cell layer and the hilus. In the dentate molecular layer, numerous immunopositive terminals making type I (Fig. 10B), and less frequently type II synapses on dendrites were seen. Terminals making synapses with dendritic spines were rarely labelled for mGluR4 (Fig. 10C). In the hilus, many dendritic profiles receiving labelled type II (Fig. 10E) and type I (Fig. 10G) synapses as well as immunopositive axon terminals on spines (Fig. 10F) were observed.

Peroxidase reaction product was occasionally found in axonal profiles in all brain areas analysed, and single immunometal particles were seen on extrasynaptic locations and dendritic spines. These patterns of labelling are difficult to interpret due to the signal being weak or rarely seen.

DISCUSSION

Specificity of antibodies to mGluR4

The antibodies raised for this study reacted specifically with mGluR4 and not with other mGluR subtypes in transfected cells. Furthermore, no immunoreactivity was detected in brains of mice carrying the gene-targeted

Fig. 6. Immunolocalisation of mGluR4-IR in the rat VP. (A) The VP, like the GP, shows intense mGluR-IR. (B) The mGluR4 immunolabelling appears as a punctate decoration of dendrites (some indicated by arrows) and somata of pallidal neurones. (C) Most of the puncta can be seen in linear arrays (arrows) surrounding dendrites. Immunogold particles (D, E, G) and peroxidase immunoreaction product (F, H), indicating mGluR4-IR, concentrate in the presynaptic membrane specialisation of type II synapses on dendritic shafts (D–H). Immunogold labelling of an axon terminal (E_1, E_2) is shown in serial sections. Abbreviations: d, dendrite; t, axon terminal. Scale bars = 0.5 mm (A); 20 μm (B); 10 μm (C); 0.2 μm (D–H).

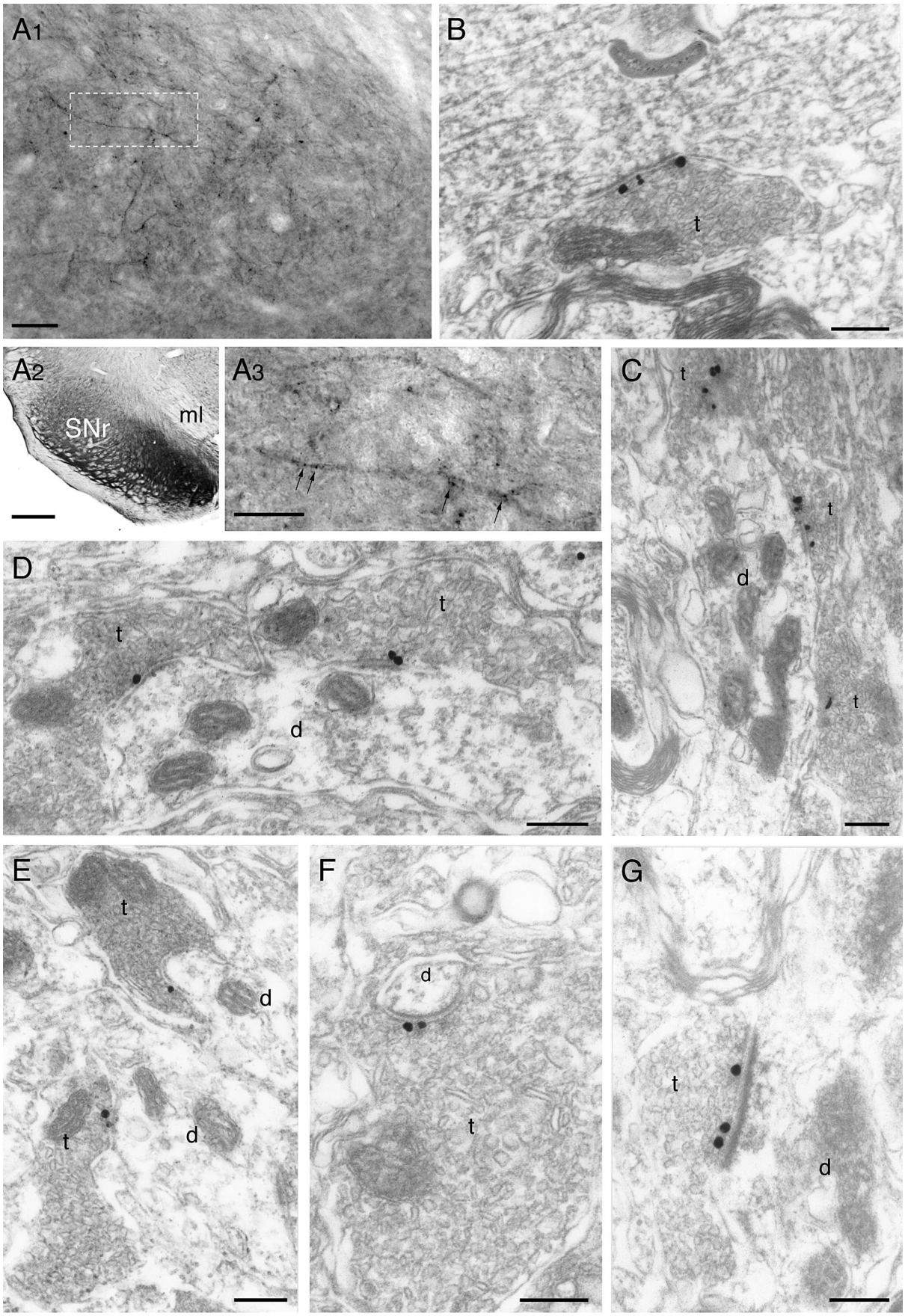

Fig. 7.

deletion of mGluR4 (Pekhletski et al., 1996), confirming the specificity of the antibodies.

The mGluR4 gene has been predicted to undergo alternative splicing generating two mGluR4 isoforms, namely mGluR4a and mGluR4b (Thomsen et al., 1997). The reported sequence of mGluR4b is identical to mGluR4a with the exception of a 620-nucleotide deletion (Thomsen et al., 1997). The resulting mGluR4b protein would thus have 135 different amino acids replacing the entire carboxyl-terminus of mGluR4a. Therefore, the detection of mGluR4b by the antibodies used in this study cannot account for the extra labelling we observed. We made numerous unsuccessful attempts to amplify by reverse transcriptase-PCR the sequence corresponding to the C-terminus of mGluR4b from several rat brain areas (cerebellar cortex, olfactory bulb, neocortex and hippocampus). On the contrary, amplification of the mGluR4a form was always achieved (C. Corti and F. Ferraguti, unpublished observations). Interestingly, other laboratories have also failed to amplify mGluR4b (M. Soloviev and R.A.J. McIhinney (MRC Anatomical Neuropharmacology Unit, Oxford University, Oxford, UK) and M. Watanabe (Department of Anatomy, Hokkaido University, Sapporo, Japan), personal communications). Recently, we have retrieved a genomic sequence (clone RP11-487B14 from the RPCI-11 Human Male BAC library; GenBank AC024449), which contains the entire complementary sequence of the human mGluR4a mRNA (X80818; Makoff et al., 1996). This allowed us to assign exon/intron boundaries within the gene and to ascertain the presence of splice site consensus sequences that could support the existence of mGluR4b transcripts. The site of deletion was present in the putative exon 9, but did not correspond to an exon/intron boundary. The human and the rat sequences, corresponding to the putative exon 9, show a 98% amino acid identity (82/83 residues) and a 90% identity at the nucleotide level, which is 100% at the deletion site. mGluR4b can only be generated by the use of an internal alternative splice site situated in the putative exon 9, but an acceptor splice site at the deletion site was not found in the genomic sequence. It should also be noted that mGluR4b, despite being obtained from the screening of a cDNA library, was only identified as a partial clone (Thomsen et al., 1997). Thus, these annotations strongly suggest that mGluR4b does not exist as a full-length transcript and that it probably represents a recombination artefact.

In both mouse and rat brain intense mGluR4-IR was found in the cerebellar cortex, basal ganglia, medial geniculate nucleus, and superficial layer of the superior colliculus, which showed a good correlation with a previously reported distribution of high density [^{3}H]L-AP4 binding sites (Hudtloff and Thomsen, 1998). Furthermore, the relative intensity of immunoreaction observed in the different brain areas tested by western blotting corresponded to mGluR4-IR observed in fixed tissue sections. Taking all the data together the results demonstrate that the antibodies react only with mGluR4.

Possible source(s) of endogenous agonists of presynaptic mGluR4

In this and previous studies, it has been shown that in the cerebellar cortex (Kinoshita et al., 1996; Mateos et al., 1998), SNr and striatum, mGluR4 is localised in presynaptic terminals making type I synaptic contacts. Hence, in these synapses mGluR4 operates as an autoreceptor, as initially postulated (Thomsen, 1997). However, in the GP, VP, SNr and entopeduncular nucleus the localisation of mGluR4 in terminals of projection neurones of both the striatum and nucleus accumbens, which are known to be GABAergic (for review see Graybiel, 1990), suggests a role for mGluR4 in regulating GABA release. Therefore, synaptically released glutamate can activate mGluR4 on the same terminal, thus acting as a presynaptic autoreceptor, as well as being able to reach neighbouring terminals and to activate mGluR4 as a presynaptic heteroreceptor. Depression of GABA release from terminals by the activation of presynaptic mGluRs has been demonstrated in cerebellar glomeruli (Mitchell and Silver, 2000) and in hippocampal CA1 interneurones (Semyanov and Kullmann, 2000). However, depression of glutamate release may also be achieved through heterosynaptic activation of presynaptic mGluRs on neighbouring glutamatergic terminals by extrasynaptic diffusion (spill-over) of glutamate. The occurrence of this type of heterosynaptic inhibition has been demonstrated at mossy fibre synapses in the hippocampus (Vogt and Nicoll, 1999). Activity-dependent dendritic release of glutamate (Zilberter, 2000) may be a further potential source of endogenous agonist at presynaptic mGluR4. Moreover, the release of an endogenous agonist other than glutamate from GABAergic terminals cannot be excluded.

Immunolocalisation of mGluR4 in the basal ganglia

We have confirmed here the prominent expression of mGluR4 in the striatopallidal projection, as previously reported by Bradley et al. (1999). However, with our antibodies intense labelling for mGluR4 was also detected in the entopeduncular nucleus, SNr and the striatum. The localisation of mGluR4 mRNA in the basal ganglia occurs only in the striatum and nucleus

Fig. 7. Immunolocalisation of mGluR4-IR in the rat SNr. (A_{1-2}) Light micrographs of mGluR4-IR in a coronal section through a midrostral level of the SNr. (A_3) The mGluR4 immunolabelling appears as punctate structures (some indicated by arrows) apposed to dendrites and somata of nigral neurones. Boxed area in A_1 is shown at higher magnification in A_3. Axon terminals immunolabelled with gold particles for mGluR4 form type II synapses with a perikaryon (B) and dendritic shafts (C–F). (E, F) Immunopositive presynaptic boutons are also seen to embrace dendritic digitations, a feature of striatal terminals. (G) Immunogold particles are also localised on the presynaptic membrane specialisation of terminals forming type I synapses with dendrites. Abbreviations: d, dendrite; ml, medial lemniscus; t, axon terminal. Scale bars = 50 μm (A_1); 0.5 mm (A_2); 20 μm (A_3); 0.2 μm (B–G).

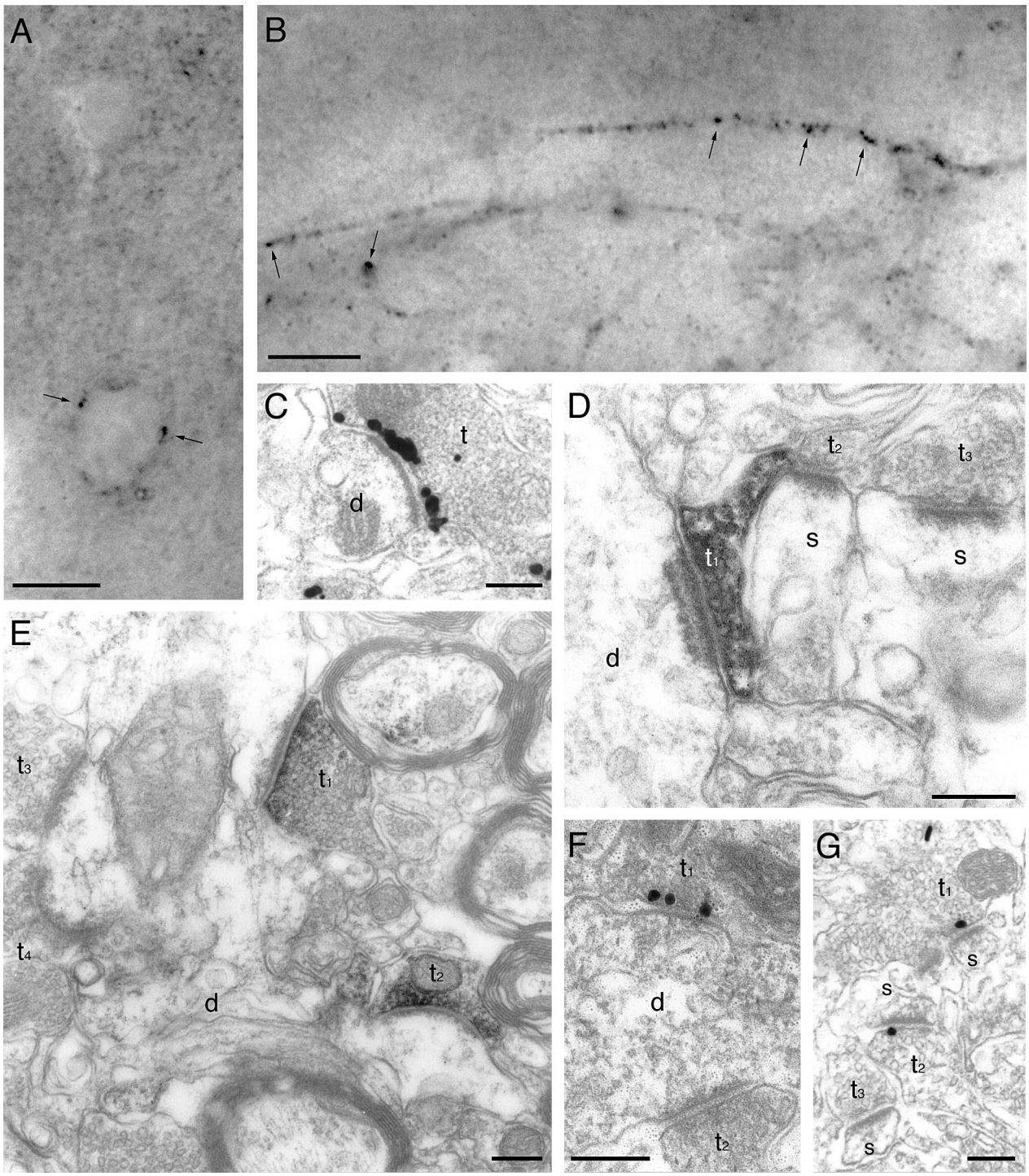

Fig. 8. Immunolocalisation of mGluR4-IR in the stratum oriens/alveus of the rat hippocampal CA1 area. (A, B) Somatodendritic profiles of interneurones in stratum oriens/alveus are apposed by intensely labelled axon terminals (some indicated by arrows). Immunogold particles (C) and peroxidase immunoreaction product (D, E), indicating mGluR4-IR, are seen in axon terminals forming type I synaptic contacts with dendritic profiles (C–E). (D) A labelled terminal (t_1), adjacent to two unlabelled boutons (t_2 and t_3) on spines, establishing a type I synapse on a dendrite is characterised by a row of postjunctional dense bodies. (E) Four type I synapses, two immunopositive (t_1 and t_2) and two immunonegative (t_3 and t_4) for mGluR4, contact a dendrite. (F) Infrequent immunolabelled terminals in type II synapses were also seen. In the example in F, a terminal (t_1) forming a type II synapse on a dendrite is labelled in the presynaptic active zone and is opposite to an immunonegative terminal (t_2) forming a type I synapse. (G) In stratum oriens/alveus occasional weakly labelled terminals on dendritic spines (t_1, t_2) were also observed. Abbreviations: d, dendrite; s, spine; t, axon terminal. Scale bars = 10 μm (A, B); 0.2 μm (C–G).

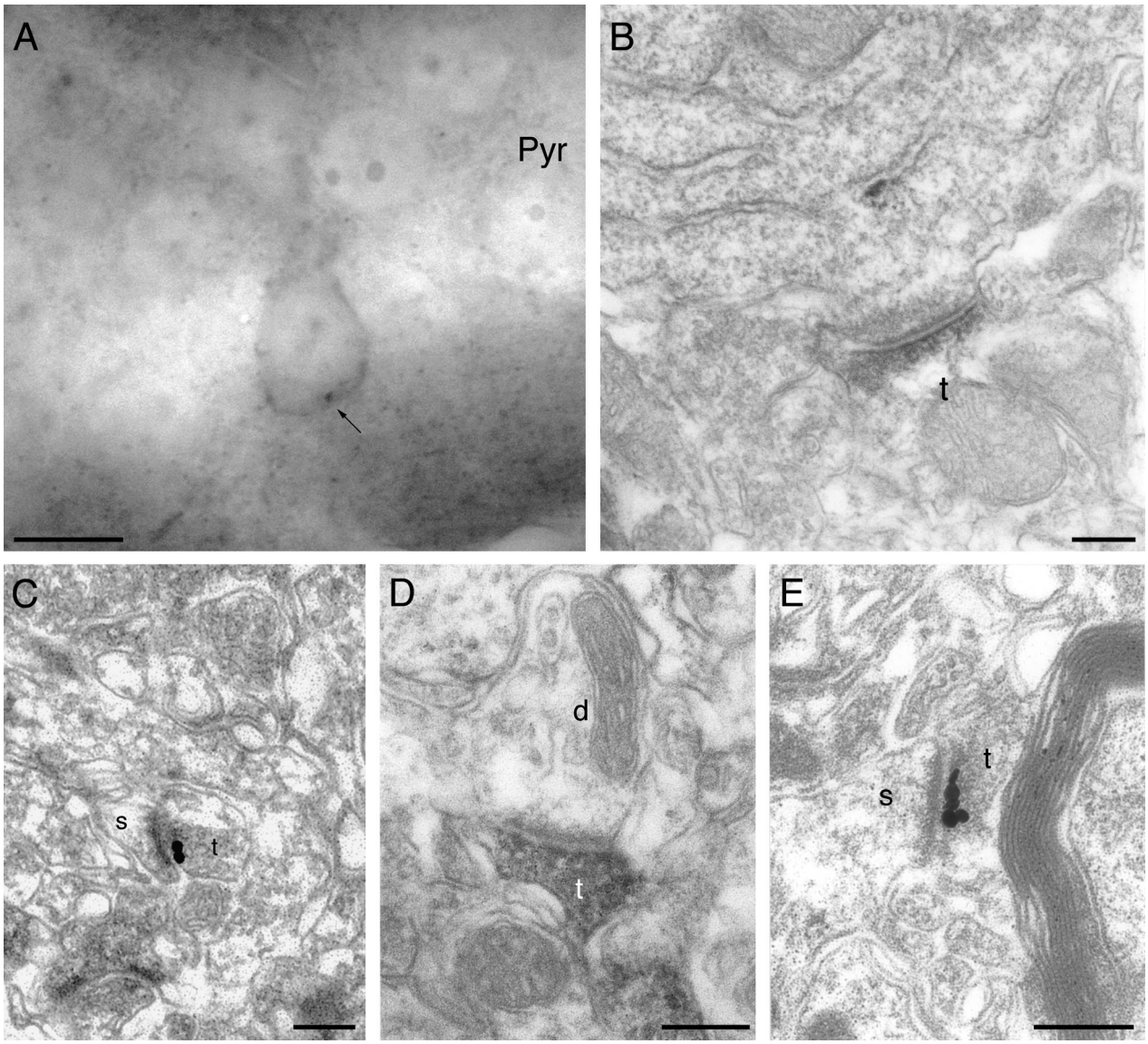

Fig. 9. Immunolocalisation of mGluR4-IR in the rat hippocampal CA1 area strata pyramidale, radiatum and lacunosum moleculare. (A) In the pyramidal cell layer (Pyr), scattered putative interneurones are outlined with a punctate decoration from labelled axon terminals (as indicated by the arrow). (B) Immunolabelled terminals on the soma of these cells form type II synaptic contacts. (C) In stratum radiatum an occasional immunolabelled terminal on a spine is shown. Stratum lacunosum moleculare contains labelled boutons forming type I synapses on (D) dendrites and (E) spines. Abbreviations: d, dendrite; s, spine; t, axon terminal. Scale bars = 10 μm (A); 0.2 μm (B–E).

accumbens, where both medium-sized and large neurones express the receptor (Ohishi et al., 1995). These cells most likely represent medium spiny neurones, which are recipients of all major afferent pathways to the striatum and are also the main source of striatal efferent axons (for review see Smith and Bolam, 1990; Parent et al., 2000). The reported limited occurrence of mGluR4 to a subset of striatopallidal enkephalin-positive terminals (Bradley et al., 1999) appears at odds with the expression of mGluR4 mRNA in the vast majority of medium-sized neurones in the striatum (Ohishi et al., 1995). Conversely, our results are consistent with the *in situ* hybridisation pattern. A possible reason why in the study of Bradley et al. (1999) only low intensity staining for mGluR4 was detected in the SNr, the entopeduncular nucleus and the striatum, could be the different antigen used for immunisations. The antibodies used in our study were raised against the entire carboxyl-terminal domain of mGluR4 (Tanabe et al., 1993), whereas Bradley et al. (1996) used only the last 19 amino acid residues. The larger polypeptide might have resulted in more or different epitopes being recognised by our antibodies, resulting in a higher sensitivity.

The presence of group III mGluRs in the striatum has been indicated by several functional studies. Application of L-AP4 to rat striatal slices was shown to give rise to a concentration-dependent biphasic inhibition of forskolin-stimulated cAMP production (Schaffhauser et al., 1997), suggesting the presence of both high and low affinity L-AP4-sensitive mGluRs. Recent immunocytochemical

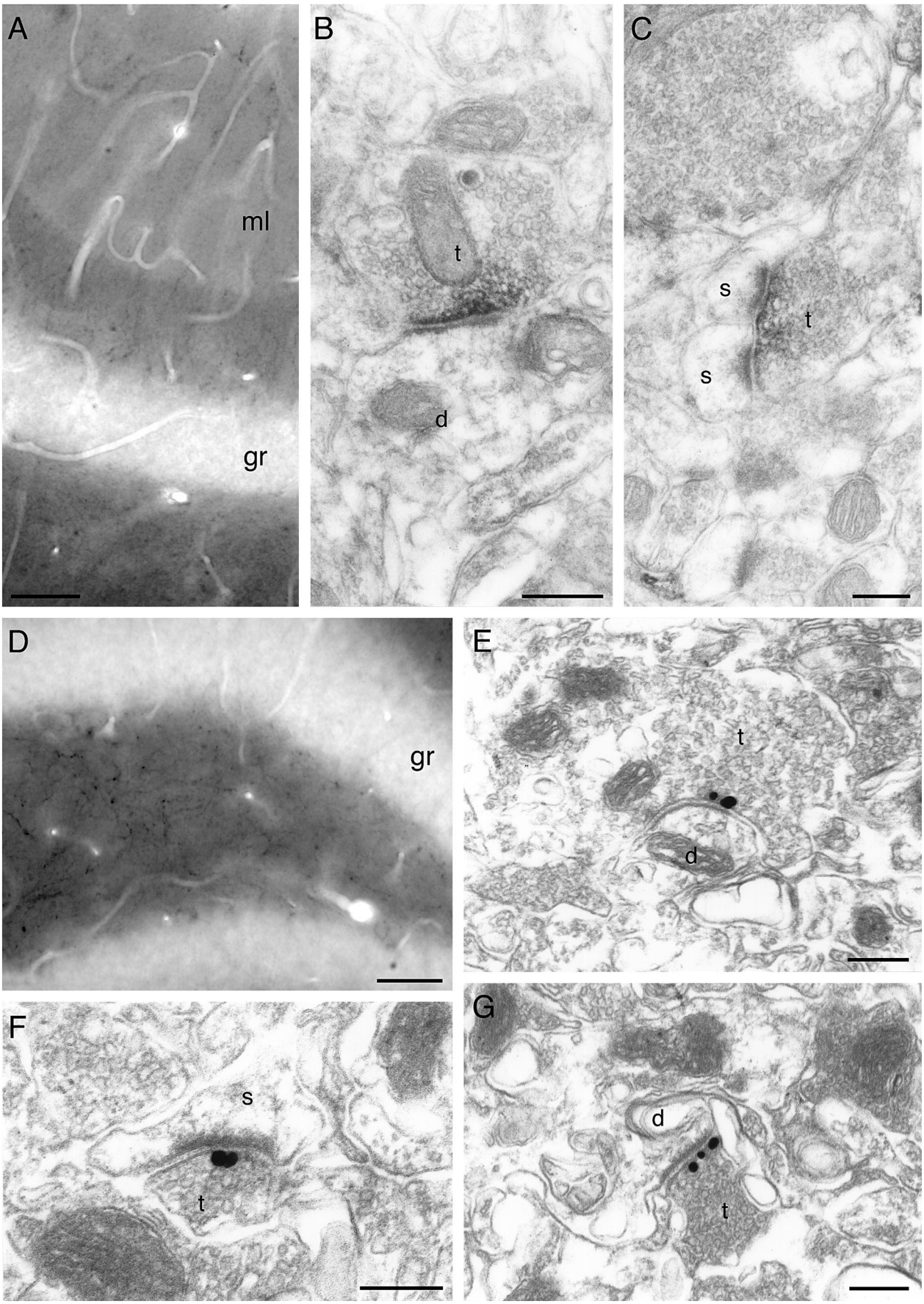

Fig. 10.

studies have confirmed the presence of mGluR7 in presumed corticostriatal terminals (Kinoshita et al., 1998; Kosinski et al., 1999), but showed a very low level of staining for mGluR4 in the striatum (Bradley et al., 1999). No reports on the distribution of mGluR8 in basal ganglia structures are yet available. However, agonists of group III mGluRs were shown to mediate depression of EPSPs at corticostriatal synapses displaying EC_{50} values consistent with the activation of mGluR4 and/or mGluR8 (Calabresi et al., 1993; Pisani et al., 1997). Our study shows that the striatum contains a significant amount of mGluR4-IR, but mostly in terminals forming type II synapses on dendrites. A relatively small number of labelled axon terminals made also type I synaptic contacts with dendritic shafts and spines of presumed projection neurones. It is possible that these axon terminals are of cortical origin, but they may also represent a component of the glutamatergic input to the striatum that arises from the thalamus (Parent and Hazrati, 1995). We also found intensely labelled terminals apposed to the dendrites of ventral pallidal neurones. These terminals formed type II synaptic contacts, and probably represent ventral striatofugal projections arising from the nucleus accumbens.

The primary glutamatergic input to the GP, VP, SNr and entopeduncular nucleus comes from the subthalamic nucleus, whose terminals converge with striatal terminals on the same dendrites (Smith et al., 1998). Therefore, it is possible that mGluR4 is activated in these areas by the extrasynaptic diffusion of glutamate from neighbouring glutamatergic synapses arising from the subthalamic nucleus.

In the SNr, the activation of group III mGluRs by L-AP4 was recently demonstrated to reduce both inhibitory (IPSCs) and excitatory postsynaptic currents (EPSCs) through a presynaptic mechanism (Wittmann et al., 2001). Inhibition of IPSCs by L-AP4, although concentration-dependent, was shown to be biphasic, thus suggesting the activation of both mGluR7 (Kosinski et al., 1999) and mGluR4/mGluR8. The presence of mGluR4 in putative GABAergic synaptic active zones suggests a contribution of this receptor in regulating transmitter release at striatonigral synapses. In the SNr the localisation of mGluR4 in type I synapses raises the question of the origin of these glutamatergic terminals. In addition to the main glutamatergic innervation from the subthalamic nucleus, which does not express mGluR4 mRNA (Ohishi et al., 1995), SNr neurones have been proposed to receive glutamatergic afferents from the mesopontine tegmentum (Bolam et al., 1991; Lavoie and Parent, 1994) and from the frontal cortex (Usunoff et al., 1982; Kornhuber et al., 1984). However, no definitive evidence has yet been provided for the existence of a corticonigral projection. Alternatively, mGluR4 may be located in non-glutamatergic excitatory terminals. One of the principal excitatory pathways which innervates the SNr arises from the serotoninergic neurones of the dorsal and median raphe nuclei (Steinbusch, 1981; Corvaja et al., 1993). No detectable levels of mGluR4 mRNA have been found in the rat pedunculopontine tegmental nucleus or in the raphe nuclei, but a weak expression of mGluR4 mRNA has been reported in the isocortex (Ohishi et al., 1995). Therefore, the putative corticonigral projection would appear as a conceivable glutamatergic input to express mGluR4. However, the presence of mGluR4 in an as yet undetected subset of neurones, e.g. in the mesopontine tegmentum, cannot be ruled out.

Localisation of mGluR4 in the hippocampal formation

High affinity [^{3}H]L-AP4 binding was primarily observed in the molecular layer of the DG, whereas other regions in the hippocampus showed very little bound radioactivity (Hudtloff and Thomsen, 1998). Similarly, in the present and a previous study (Shigemoto et al., 1997), mGluR4-IR was primarily detected in the neuropil of the inner third of the molecular layer of the DG, which corresponds to the primary terminal field of the associational/commissural pathway (see Amaral and Witter, 1995). Perforant path terminal zones were only weakly labelled. Consistent with these findings, L-AP4-mediated inhibition of perforant path inputs to the DG (Koerner and Cotman, 1981) was reported to be independent of mGluR4 activation (Pekhletski et al., 1996). In addition to perforant path inputs, excitatory synaptic inputs to hilar–dentate border interneurones were also reversibly depressed by low doses of L-AP4 (Doherty and Dingledine, 1998). Glutamatergic innervation of hilar–dentate border interneurones, within the hilus, mainly originates from dentate granule cells and CA3 pyramidal cells (Frotscher, 1989; Deller et al., 1996; Geiger et al., 1997; Acsady et al., 1998), both of which express mGluR4 mRNA (Ohishi et al., 1995). Our finding that numerous dendritic shafts in both the hilus and dentate molecular layer were decorated with mGluR4-labelled terminals supports a contribution of mGluR4 in the L-AP4-mediated depression of synaptic activity at dentate– and CA3–hilar synapses on interneurones. Activation of mGluR4 on these synapses may regulate the excitatory drive of GABA releasing interneurones, which mainly innervate principal cells.

In the hilus, we also found the presence of mGluR4 immunolabelling in axon terminals forming type II synapses on dendritic shafts. Although the identity of the postsynaptic target cells could not be established, mGluR4-

Fig. 10. Immunolocalisation of mGluR4-IR in the rat DG. (A) Light micrograph of mGluR4-IR in a coronal section of the DG in which the inner third of the molecular layer (ml) appears more intensely stained. Peroxidase immunoreaction product indicating mGluR4-IR occurs in axon terminals forming type I synaptic contacts with a dendritic shaft (B) and spines (C). (D) In the hilus, a network of somatodendritic profiles is apposed by granules showing mGluR4-IR. Axon terminals immunolabelled with gold particles for mGluR4 form type II synapses with a dendritic profile (E), and type I synapses with a spine (F) and a dendritic shaft (G). Abbreviations: d, dendrite; gr, granule cell layer; s, spine; t, axon terminal. Scale bars = 50 μm (A, D); 0.2 μm (B, C, E–G).

positive axon terminals are most likely to originate from other hilar interneurones, as most hilar neurones appear to express mGluR4 mRNA (Ohishi et al., 1995).

Electrophysiological experiments have demonstrated an L-AP4-mediated depression of GABAergic synaptic transmission among CA1 hippocampal interneurones (Kogo et al., 1999; Semyanov and Kullmann, 2000). The effectiveness of L-AP4 suggested activation of mGluR4 and/or mGluR8. Our observations corroborate these findings by showing mGluR4-labelled axon terminals establishing type II synapses with dendritic profiles of CA1 stratum oriens/alveus interneurones. The interneurones in stratum oriens receive a significant innervation from local pyramidal cells. EPSCs recorded from CA1 stratum oriens/alveus interneurones were shown to be reversibly depressed by L-AP4 through a presynaptic action (Scanziani et al., 1998). The somatodendritic profiles of one class of interneurones expressing mGluR1a were previously shown to be decorated with terminals intensely labelled for mGluR7a and making type I synapses (Shigemoto et al., 1996, 1997). Like the depression of inhibitory postsynaptic currents, the potency of L-AP4 in evoking depression of EPSCs is inconsistent with the known EC_{50} on mGluR7 (Okamoto et al., 1994), suggesting that presynaptic group III mGluRs other than mGluR7a are involved. This conclusion is confirmed by the detection of strong mGluR4-IR in terminals of type I synapses on dendrites of some interneurones reported in this study. At present we cannot exclude the possibility that mGluR8 also contributes to the effects of L-AP4 (Scanziani et al., 1998; Kogo et al., 1999; Semyanov and Kullmann, 2000).

It is not apparent from the present results which afferent(s) to CA1 stratum oriens/alveus interneurones contain mGluR4 at their terminals, nor could we identify the specific classes of interneurone receiving mGluR4-enriched input. Further studies will be needed to clarify these important issues.

CONCLUSIONS

The results demonstrate that, like other group III mGluRs, presynaptic mGluR4 is highly enriched in the active zone of terminals innervating specific neurones. The localisation of mGluR4 in striatopallidal and striatonigral terminals, where it probably regulates GABA release acting as a presynaptic heteroreceptor, supports a principal role for this receptor in the depression of synaptic transmission produced by L-AP4 in the basal ganglia. Similarly, in the hippocampus, the results imply a regulation of GABAergic transmission and of the excitatory synaptic input to interneurones by mGluR4. The highly discrete localisation observed in the hippocampus suggests a regulation of synaptic transmission through specific circuits, which are probably dependent on the differential density of the receptor to synapses on specific cell targets.

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