

# Altered expression patterns of group I and II metabotropic glutamate receptors in multiple sclerosis

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## Summary

Recent evidence supports a role for glutamate receptors in the pathophysiology of multiple sclerosis. In the present study, we have focused specifically on the expression of metabotropic glutamate receptors (mGluRs) in multiple sclerosis brain tissue. The expression of group I (mGluR1 $\alpha$  and mGluR5) and group II (mGluR2/3) mGluRs was studied using immunohistochemistry in tissue from 12 multiple sclerosis cases and seven non-neurological controls. The expression patterns of both group I and II mGluRs in multiple sclerosis tissue differed significantly from those in control tissue. Strong mGluR1 $\alpha$  immunoreactivity was observed in axons of the subcortical white matter, particularly in the centre of actively demyelinating lesions and in the borders of chronic active lesions. mGluR1 $\alpha$  axonal immunopositivity was also found in normal appearing multiple sclerosis white matter, but axons in control white matter were generally negative. mGluR1 $\alpha$  axonal labelling

was associated with the presence of non-phosphorylated neurofilaments and  $\beta$ -amyloid precursor protein, which are sensitive markers for axonal injury and disturbed axonal transport. Changes in mGluR immunoreactivity were also observed in glia. A diffuse increase in the expression of mGluR5 and mGluR2/3 was detected in reactive astrocytes in multiple sclerosis lesions. However, only a subpopulation of reactive astroglial cells expressed mGluR1 $\alpha$ . In addition, labelling with antibodies to mGluR2/3 and, to a lesser extent labelling with antibodies to mGluR1 $\alpha$ , was detected in a population of cells of the microglial/macrophage lineage that displayed a macrophage-like morphology. Our data suggest that mGluRs, like ionotropic glutamate receptors, play a role in the complex processes that are associated with the progressive brain damage in multiple sclerosis, including both glial activation and pathological changes in axons.

**Keywords:** metabotropic glutamate receptors; reactive astrocytes; axons; microglia; macrophages.

**Abbreviations:** Ab = antibody;  $\beta$ -APP =  $\beta$ -amyloid precursor protein; EAE = experimental autoimmune encephalomyelitis; GFAP = glial fibrillary acidic protein; IR = immunoreactivity; mAb = monoclonal antibody; mGluR = metabotropic glutamate receptor; MOG = myelin oligodendrocyte glycoprotein; NAWM = normal appearing multiple sclerosis white matter; PP = primary progressive; SP = secondary progressive

## Introduction

Multiple sclerosis is a chronic demyelinating disease of the CNS, which primarily affects young adults and in most cases leads to chronic disability. Little is known yet about the pathogenesis of the disease. Several hypotheses have been proposed, including viral infection and autoimmunity (Meinl, 1999; Noseworthy, 1999; Lucchinetti *et al.*, 2000; Simmons, 2001; Gutierrez *et al.*, 2002; Levin *et al.*, 2002). Recent

studies have suggested a possible role for glutamate excitotoxicity in the pathophysiology of this disease (for a review see Matute *et al.*, 2001). Patients with multiple sclerosis were found to have increased CSF levels of excitatory amino acids (Stover *et al.*, 1997; Barkhatova *et al.*, 1998), and alterations in glutamate homeostasis have been observed in multiple sclerosis lesions (Werner *et al.*,

2001). In addition, correlations between altered glutamate homeostasis and oligodendrocyte and axonal damage have been reported in animals with experimental autoimmune encephalomyelitis (EAE, an animal model for multiple sclerosis) (Matute *et al.*, 1999, 2001; Werner *et al.*, 2001). Oligodendroglial cells have been shown to be highly vulnerable to glutamate-mediated toxicity both *in vitro* and *in vivo* (Matute *et al.*, 1997; McDonald *et al.*, 1998). This, together with the observation that treatment with a specific glutamate receptor antagonist results in a substantial improvement of the disease in animals with EAE (McDonald *et al.*, 1998), strongly supports the involvement of glutamate and its receptors in the pathogenesis of demyelinating disorders.

Previous studies have focused specifically on the role of ionotropic glutamate receptors, which are ligand-gated ion channels [including *N*-methyl-D-aspartate (NMDA), kainate and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors] in the pathogenesis of multiple sclerosis (Matute, 1998; Pitt *et al.*, 2000; Paul and Bolton, 2002). However, the cell-specific distribution and the role of metabotropic glutamate receptors (mGluRs) in multiple sclerosis tissue have not yet been defined.

mGluRs consist of at least eight subtypes and they regulate a variety of intracellular signalling systems via activation of GTP-binding proteins (Pin and Duvoisin, 1995; Schoepp *et al.*, 1999). They are subdivided into three main groups. Group I includes mGluR1 and -5, which are coupled to phosphoinositide hydrolysis, while group II (mGluR2 and -3) and group III (mGluR4, -6, -7 and -8) mGluRs are negatively coupled to adenylyl cyclase (Schoepp *et al.*, 1999).

Depending on the receptor subtype, mGluRs are localized at the postsynaptic and/or presynaptic sites of neuronal elements, as well as in glial cells (Cartmell and Schoepp, 2000). Neuronal mGluRs are thought to play important roles in synaptogenesis and synaptic plasticity, and also in several pathophysiological processes (for reviews see Miller *et al.*, 1995a; Bordi and Ugolini, 1999). The neuronal localization of mGluRs to the somato-dendritic and/or axonal compartments is particularly interesting in relation to the morphological and functional axonal changes described in multiple sclerosis (Ferguson *et al.*, 1997; Trapp *et al.*, 1998; for a review see Kornek and Lassmann, 1999). Axonal injury in multiple sclerosis has been demonstrated within active lesions, in association with activation of microglia/macrophages and astroglia, and in normal-appearing white matter (NAWM) (Giordana *et al.*, 2002; Rieckmann and Maurer, 2002).

Glial cells also express mGluR subtypes (Romano *et al.*, 1995; Petralia *et al.*, 1996; Biber *et al.*, 1999; Aronica *et al.*, 2000, 2001a; Taylor *et al.*, 2002). The astroglial expression of both group I and II subtypes appears to be dynamic, with expression levels changing in response to different types of brain injury *in vivo* (Boxall *et al.*, 1996; Ulas *et al.*, 2000; Aronica *et al.*, 2000, 2001a, b; Ferraguti *et al.*, 2001) and upon exposure of cultured astrocytes to growth factors (Miller

*et al.*, 1995b; Minoshima and Nakanishi, 1999; Aronica *et al.*, 2002). Several observations suggest that glial mGluRs can regulate glial function and may be involved in the interaction between glia and neurons in both physiological and pathological conditions (Chiu and Kriegler, 1994; Ciccarelli *et al.*, 1997; Agrawal *et al.*, 1998; Bruno *et al.*, 1998; McGeer and McGeer, 1998; Aronica *et al.*, 2002; Taylor *et al.*, 2002). Moreover, glial mGluRs may play a role in neuroinflammatory disorders as specific mGluR agonists have the ability to regulate the production of chemokines RANTES by astrocytes *in vitro* and to improve the rate of functional recovery from EAE *in vivo* (Besong *et al.*, 2002).

In the present study, immunocytochemistry with antibodies (Abs) specific for mGluR1 $\alpha$ , mGluR2/3 and mGluR5 was performed on post-mortem control and multiple sclerosis brain samples. Our aim was to define the possible involvement of mGluR in the pathophysiology of multiple sclerosis, and increase the knowledge on the role of excitotoxicity in this disease.

## Material and methods

### Subjects and tissue

Tissues from the 12 multiple sclerosis and seven control cases that were used in this study were obtained from of the Departments of Pathology of the Vrije Universiteit (VU) Medical Center and the Academic Medical Center (University of Amsterdam) and from the Netherlands Brain Bank (coordinator, R. Ravid). The Netherlands Brain Bank received permission to perform autopsies, for the use of tissue and for access to medical records for research purposes from the Ethical Committee of the VU Medical Center, Amsterdam, The Netherlands. Patients were rapidly autopsied and tissue samples were selected on the basis of post-mortem MRI. The tissue samples used in this study were selected on the basis of the presence of both white and cortical grey matter in the section. Most of the samples had at least one multiple sclerosis lesion in or in the proximity of the grey matter. Areas of NAWM were also selected. All cases were reviewed independently by two neuropathologists. The diagnosis of multiple sclerosis and the classification of the lesions followed the previously described histopathological criteria (van der Valk and De Groot, 2000). The classification of the lesions was based on extensive analysis of the distribution of inflammatory cells, T cells, macrophages and astrocytes, and on the presence of active myelin breakdown, as previously reported (van der Valk and De Groot, 2000; Hulshof *et al.*, 2002). The clinical features (derived from patients' medical records) of the multiple sclerosis and controls subjects are summarized in Table 1.

### Tissue preparation

Paraffin-embedded tissue was sectioned at 5  $\mu$ m and mounted on organosilane (3-aminopropylethoxysilane;

**Table 1** Summary of the clinical and neuropathological details of the multiple sclerosis and control patients

Case	Gender	Age (years)	Post-mortem delay (h)	Duration of clinical symptoms (years)	Diagnosis (disease type)
1	F	53	11	27	MS (SP)
2	F	64	7.5	34	MS (SP)
3	F	48	8	8	MS (SP)
4	F	40	7	11	MS (SP)
5	F	52	8.5	22	MS (SP)
6	M	69	10	34	MS (PP)
7	M	43	8.5	11	MS (SP)
8	F	35	5.5	11	MS (SP)
9	F	53	7.5	18	MS (SP)
10	F	63	8	24	MS (SP)
11	F	34	10	10	MS (SP)
12	F	82	8	23	MS (PP)
Mean $\pm$ SEM		53 $\pm$ 4	8.3 $\pm$ 0.4	19 $\pm$ 3	
13	F	82	5.5		NC
14	M	88	7.5		NC
15	M	80	11		NC
16	F	42	5.5		NC
17	M	54	6		NC
18	M	76	6		NC
19	F	35	10.5		NC
Mean $\pm$ SEM		65 $\pm$ 8	7.4 $\pm$ 0.9		

MS = multiple sclerosis; SP = secondary progressive; PP = primary progressive; NC = normal control; SEM = standard error of the mean.

Sigma Chemical Company, St Louis, MO) coated slides. Representative sections of all specimens were processed for standard haematoxylin–eosin and Luxol Fast Blue staining, as well as for immunocytochemical reactions using a number of neuronal and glial markers described below. Sequential sections of 5  $\mu$ m were also used to study the distribution of neurofilaments, vimentin, glial fibrillary acidic protein (GFAP) and mGluRs within the same specimen. For indirect immunofluorescence labelling studies, multiple sclerosis material was fixed in a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4), for 1–7 days at 4°C and then incubated in a solution of 30% sucrose for 1–3 days at 4°C under constant rotation (Wolswijk, 1998, 2002). The tissue subsequently was placed in a boat prepared from aluminium foil containing Tissue-Tek OCT embedding compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands), frozen on dry ice and stored at –80°C. Sections (10  $\mu$ m thick) were cut from each tissue block using a Reichert–Jung 2800 cryostat (cutting temperature: –25°C), mounted onto SuperFrost\*Plus microscope slides (Menzel-Gläser, Germany), stored at –20°C and then immunolabelled using indirect immunofluorescence techniques, as described below.

### Antibody characterization

Abs against GFAP (polyclonal rabbit, DAKO A/S, Glostrup, Denmark; 1 : 2000; monoclonal mouse, Chemicon

International, Inc., Temecula, CA, 1 : 50), vimentin [monoclonal Ab (mAb), clone V9, DAKO; 1 : 100], human leukocyte antigen (HLA)-DP, DQ, DR (mAb IgG<sub>1</sub>; DAKO, 1 : 300), myelin oligodendrocyte glycoprotein (MOG; the Z12 IgG<sub>2a</sub> mAb, 1 : 300; a generous gift of Dr L. Diemel; Piddlesden *et al.*, 1993), neurofilaments (mAb clone 2F11, DAKO, 1 : 500; mAb clone RT97 IgG<sub>1</sub>, 1 : 300, Chemicon; mAb SMI-32, IgG<sub>1</sub>, 1 : 300, Sternberger Monoclonals, Inc., MD) and  $\beta$ -amyloid precursor protein (anti- $\beta$ -APP mAb; Boehringer Mannheim, Germany; 5  $\mu$ g/ml) were used in the routine immunocytochemical analysis to document the presence of reactive gliosis and axonal damage in the multiple sclerosis tissue. The SMI-32 antibody (against non-phosphorylated neurofilament) provides a sensitive marker for axonal pathological changes (Trapp *et al.*, 1998). In normal and lesion areas, resting astrocytes were distinguished from reactive astrocytes on the basis of their morphology and the absence of vimentin immunoreactivity (IR), as previously reported (Khurgel and Ivy, 1996; Aronica *et al.*, 2000).

For the detection of mGluRs, we used Abs specific for the mGluR subtypes 1 $\alpha$ , 2/3 (polyclonal rabbit, Chemicon; 1 : 100) and 5 (polyclonal rabbit, Upstate Biotech, Lake Placid, NY, 1 : 100). Characterization of these Abs in human brain tissue has been documented previously (Aronica *et al.*, 2001a, b). The Ab specificity was tested by pre-incubating the Abs with 100-fold excess of the antigenic peptides and by western blots of the total homogenates of human control brain.

### Immunohistochemistry

For single-label immunocytochemistry, sections were deparaffinated in xylene, rinsed in descending concentrations of ethanol (100, 97 and 70%) and incubated for 20 min with 1% H<sub>2</sub>O<sub>2</sub> diluted in methanol. Slides were then washed with PBS (10 mM, pH 7.4). The slides were placed into sodium citrate buffer (0.01 M, pH 6.0) and heated for 10 min in a microwave oven (650 W), with the exception of immunolabellings involving GFAP. The slides were allowed to cool for 20 min in the same solution at room temperature and then washed in PBS. They were incubated with a mixture of 10% normal goat serum (NGS), for 1 h prior to the incubation with the primary Ab (30 min at room temperature and at 4°C overnight). Sections were then washed thoroughly with PBS and incubated at room temperature for 1 h with the appropriate biotinylated secondary Ab diluted in PBS (1 : 400 goat-anti rabbit Ig or 1 : 200 goat-anti mouse Ig; DAKO). Single-label immunocytochemistry was carried out using an avidin–biotin peroxidase method (Vector Elite) with 3,3'-diaminobenzidine (DAB) as a chromogen. Sections were counterstained with haematoxylin, dehydrated in alcohol and xylene and coverslipped. Sections incubated without the primary Ab or with pre-immune sera were essentially blank.

Immunofluorescence labelling studies were carried out on 5 µm thick paraffin-embedded tissue and on 10 µm thick cryostat sections cut from 4% paraformaldehyde-fixed tissue blocks, as described in detail previously (Wolswijk, 1998, 2002). Sections were incubated for 3 days at 4°C in the primary Ab solutions (diluted in Tris-buffered saline containing 0.125% Triton X-100 and 2.5% heat-inactivated calf serum), rinsed several times in Tris-buffered saline (pH 7.6) and then incubated for 2 days at 4°C with the fluorochrome [fluorescein isothiocyanate (FITC) or Cy3]-conjugated or biotinylated anti-rabbit or anti-mouse Ig subclass-specific Abs (1 : 200; purchased from either Southern Biotechnology Associates, Inc., Birmingham, AL, or Jackson Immuno-Research, West Grove, PA). The binding of the biotinylated Abs was visualized by incubating sections for 2 days at 4°C in the presence of the Cy5-coupled streptavidin (1 : 100; Vector Laboratories,). Sections were viewed on a Zeiss Axiophot microscope equipped with phase-contrast, Nomarski, bright-field and dark-field optics, epi-UV illumination and selective filters optimized for distinguishing between FITC, Cy3 and Cy5 emission, or on a Zeiss 410 inverted confocal laser scanning microscope equipped with lasers emitting at 488, 543 and 633 nm to excite FITC, Cy3 and Cy5, respectively.

### Evaluation of immunostaining

All labelled tissue sections were evaluated by two independent observers, with respect to the presence or absence of various histopathological parameters and specific IR for the different markers. Two representative paraffin sections per case were stained and assessed with each Ab. The intensity of mGluR immunoreactive staining was evaluated using a scale

of 0–3 (0: –, no; 1: +/-, weak; 2: +, moderate; 3: ++, strong staining). The frequency of mGluR1α-positive axons and mGluR1α- (-5, -2/3) positive astroglial cells [(1) rare; (2) sparse; (3), high] was also evaluated to give information about the relative number of mGluR-positive axonal or astroglial structures within the multiple sclerosis tissue.

The product of these two values (intensity and frequency scores) was taken to give the overall score (total score) shown in Fig. 3.

## Results

### Details of multiple sclerosis and control cases

The clinical features of the 12 multiple sclerosis cases included in this study are summarized in Table 1. At death, all multiple sclerosis patients had persistent neurological deficits and the length of multiple sclerosis history ranged from 8 to 34 years (mean: 19.4 years). In 10 patients, a secondary progressive (SP) disease course was observed, while two patients had the primary progressive (PP) form of multiple sclerosis.

None of the control subjects had confounding neurological or neuropathological abnormalities.

### Metabotropic glutamate receptor expression in multiple sclerosis brain tissue

To investigate the possible changes of neuronal and glial mGluR expression in multiple sclerosis brain, we performed an immunocytochemical study for both group I (mGluR1α, mGluR5) and group II (mGluR2/3) receptors on active, chronic active and chronic inactive lesions. We also examined normal appearing multiple sclerosis tissue for changes in IR that are independent of the acute inflammatory process associated with demyelination.

In multiple sclerosis cortical grey matter, the cellular pattern of mGluR1α, mGluR5 and mGluR2/3 was similar to that observed in the control specimens and to that reported previously for human control cerebral tissue (Ong *et al.*, 1998; Oka and Takashima, 1999; Aronica *et al.*, 2001b; Tang and Lee, 2001; Tang *et al.*, 2001).

However, in the multiple sclerosis lesions and in the NAWM, the mGluR expression levels, as well as the pattern of mGluR IR differed from those in control tissue.

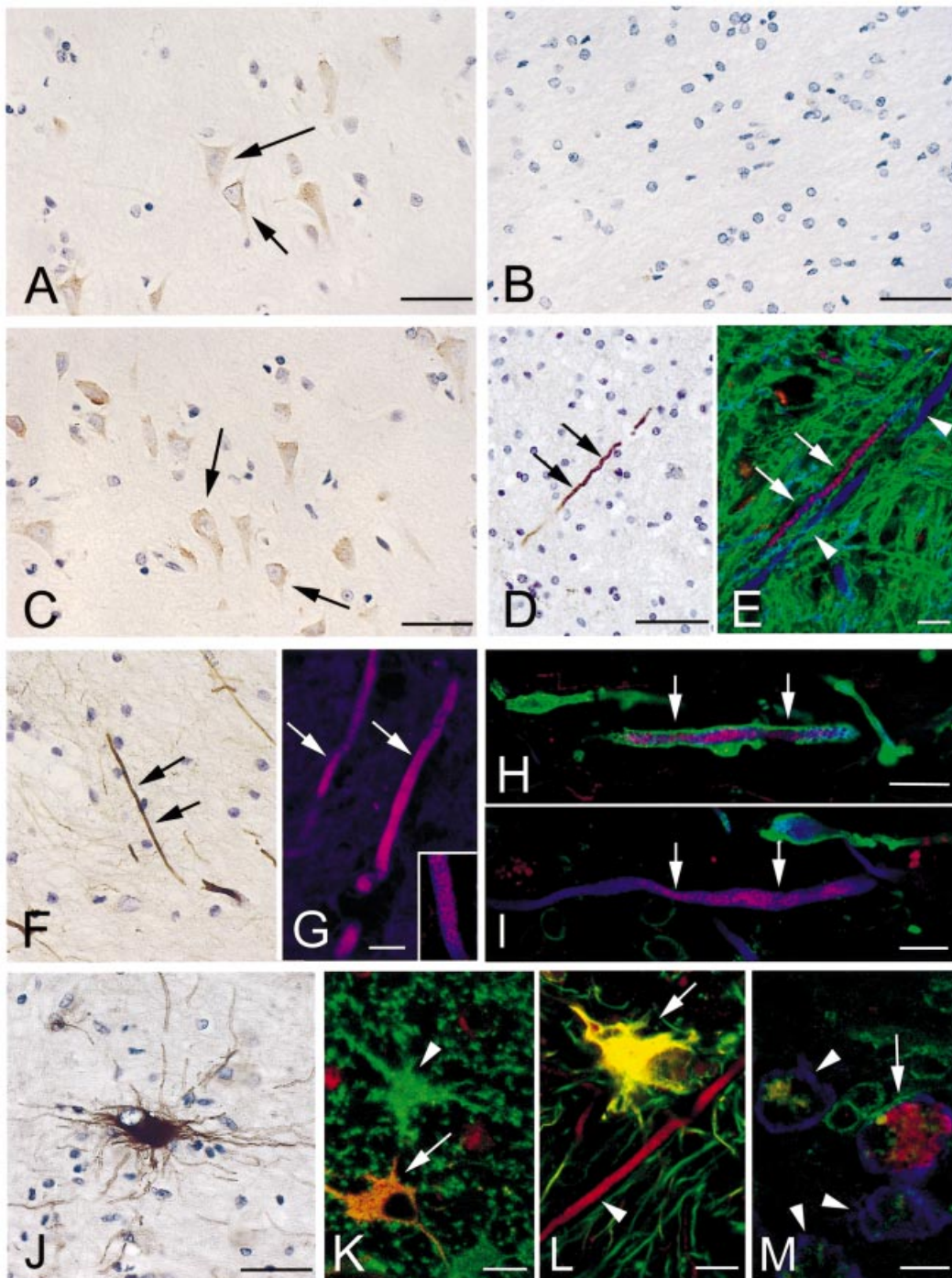
### Changes in mGluR1α expression pattern: axonal immunoreactivity

In agreement with previous observations in human control specimens (Ong *et al.*, 1998; Aronica *et al.*, 2001b), mGluR1α IR was present mainly in neurons with a diffuse somatodendritic distribution and an occasionally punctate labelling in the cellular membrane (Fig. 1A). mGluR1α IR was not detected in glial cells in control cortex (Fig. 1A) and subcortical white matter (Fig. 1B). In addition, axonal

staining was not observed in control brain (Fig. 1A and B). The overall pattern of staining in multiple sclerosis cortex tissue was comparable with that observed in control tissue (Fig. 1C). In contrast, in the NAWM of the six multiple sclerosis specimens studied, we observed axonal mGluR1 $\alpha$  IR (Fig. 1D and E; Table 2). As previously described, axonal pathological changes in multiple sclerosis tissue can be detected using Abs against non-phosphorylated neurofilaments (the SMI-32 antibody; Trapp *et al.*, 1998). Triple immunolabelling studies showed that some myelinated SMI-

32-positive axons in NAWM of multiple sclerosis specimens displayed mGluR1 $\alpha$  labelling (Fig. 1E).

Axonal localization of mGluR1 $\alpha$  was also observed in demyelinated areas. Both active and chronic active lesions contained axons with variable amounts of mGluR1 $\alpha$  (Figs 1F–I and 3A; Table 2). In the borders of active multiple sclerosis lesions, triple-labelling experiments involving Abs to mGluR1 $\alpha$ , MOG and SMI-32 revealed the presence of mGluR1 $\alpha$  IR in both partially and completely demyelinated SMI-32-positive axons (Fig. 1H and I). Although not all SMI-



32-positive axons were positive for mGluR1 $\alpha$  (Fig 1E), no mGluR1 $\alpha$  IR was detected in axons stained with Abs to phosphorylated neurofilaments (the Abs 2F11 and RT97; not shown).

Double-labelling of sections with Abs to mGluR1 $\alpha$  and  $\beta$ -APP (a molecule enriched in axons with a disturbance in axonal transport; Ferguson *et al.*, 1997; Kornek *et al.*, 2000; Bitsch *et al.*, 2000; Kuhlmann *et al.*, 2002) showed co-localization in some axons (Fig. 1G).

### Changes in mGluR immunoreactivity in glia

All multiple sclerosis lesions examined showed prominent astrogliosis. As previously described (Hulshof *et al.*, 2002), GFAP-positive astrocytes were evenly distributed throughout the demyelinated area in both active lesions and chronic active plaques. Reactive astrocytes were large cells and displayed an increased expression of vimentin and GFAP (Figs 1J–L and 2C–G).

In agreement with our previous observations in human brain tissue (Aronica *et al.*, 2000, 2001b, 2002), mGluR5 and mGluR3 were the two main mGluR subtypes expressed by astrocytes, in both physiological and pathological conditions. Control specimens contained weak to moderate mGluR5- and mGluR2/3-positive astroglial cells, most notably in the subcortical white matter (Fig. 2A and B). A diffuse upregulation of mGluR5 and mGluR2/3 IR was, however, observed in astrocytes present in both acute and chronic active multiple sclerosis lesions (Fig. 2C and D). The vast majority of the GFAP/vimentin-positive reactive astrocytes showed strong membranous and cytoplasmic staining for both receptor types (Fig. 2E–G). No detectable mGluR1 $\alpha$  IR was observed in glial cells of control cortex and white matter (Fig. 1A and B). However, mGluR1 $\alpha$  IR was observed in a subpopulation of reactive astrocytes present in demyelinated areas (Fig. 1J–L). Figure 3B–D shows the distribution of mGluR astroglial IR (total) score in control and multiple sclerosis tissue.

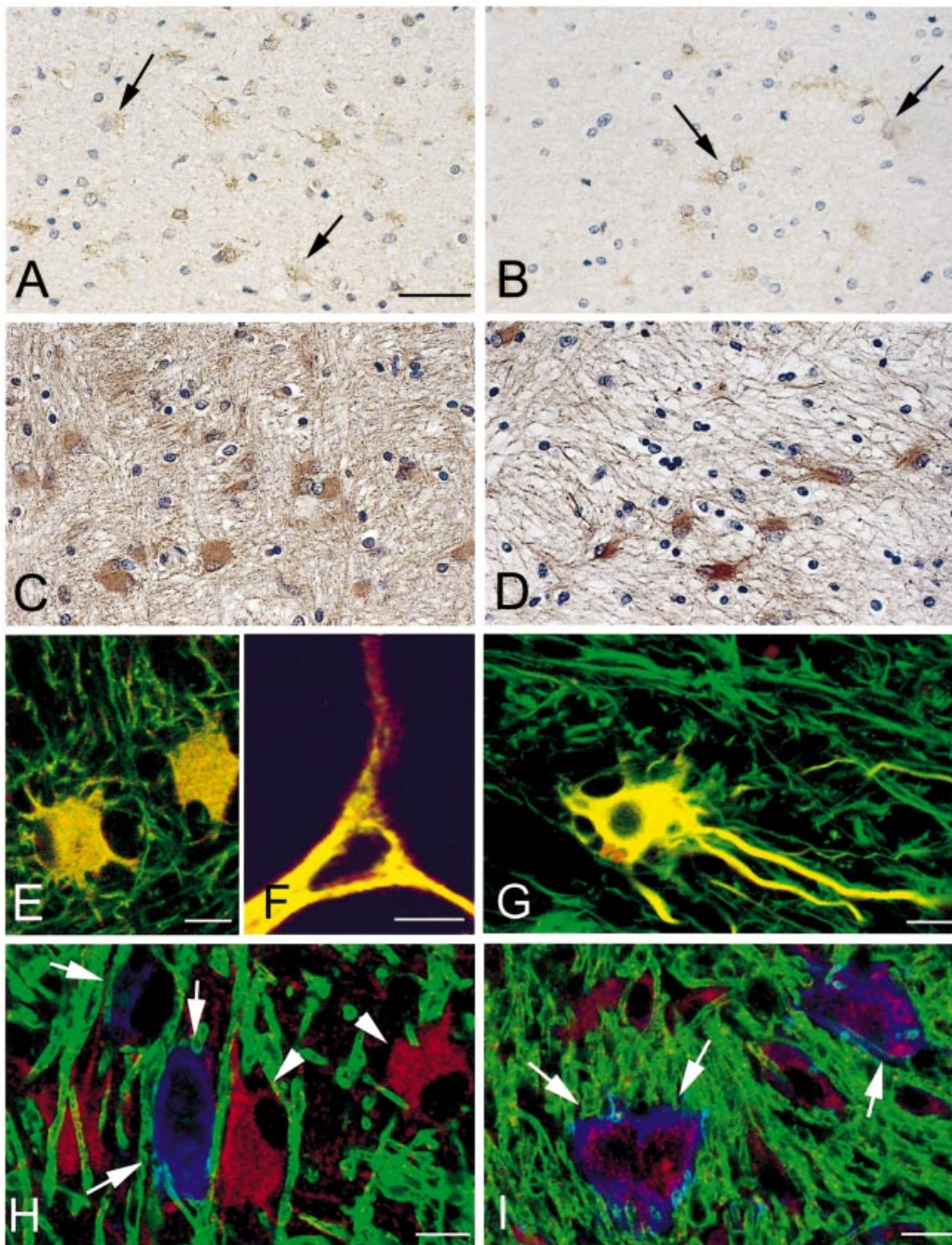
The inflammatory response in multiple sclerosis also involves cells of the microglial/macrophage lineage. Such cells are thought to play key regulatory and pathophysiological roles in the pathogenesis of this disorder (Carson, 2002). In control human brain, no detectable mGluR-1, -5 and -2/3 IR was observed in cells with the morphology of resting microglia [Figs 1A and B, and 2A and B; Aronica *et al.*, 2001a). Since mGluRs are expressed in microglial cells activated in culture (Biber *et al.*, 1999; Taylor *et al.*, 2002),

**Table 2** Summary of mGluR1 $\alpha$  immunoreactivity in axons in multiple sclerosis and control tissue

Case	Tissue	mGluR1 $\alpha$ IR	
		Intensity	Frequency
1	NAWM	+	(2)
	Chronic active lesion	++	(2)
	Active lesion	++	(1)
2	Active lesion	++	(3)
3	NAWM	+/-	(1)
4	Active lesion	++	(2)
5	Chronic active lesion	+	(1)
6	NAWM	+	(1)
	Chronic inactive lesion	+	(1)
7	NAWM	++	(1)
	Chronic active lesion	+	(1)
	Active lesion	++	(2)
8	Active lesion	++	(2)
	Chronic active lesion	++	(2)
9	Active lesion	+	(2)
10	Chronic active lesion	++	(3)
11	NAWM	++	(1)
	Active lesion	++	(3)
12	NAWM	+	(1)
	Chronic inactive lesion	+	(1)
13–19	Control tissue	-	

Immunoreactivity (IR) in axons. Intensity of the staining: -, no; +/-, weak; +, moderate; ++, strong. Frequency of immunopositive axons: (1) rare; (2) sparse; (3) high.

**Fig. 1** Distribution of mGluR1 $\alpha$  immunoreactivity (IR) in control (A and B) and multiple sclerosis (C–M) brain tissue. (A) Control cortex with a diffuse neuronal somatodendritic distribution of mGluR1 $\alpha$  IR (arrows). Control white matter (B) is almost completely unstained. In multiple sclerosis cortical grey matter (C), the cellular pattern was not obviously different from the pattern of mGluR1 $\alpha$  staining in control sections. In multiple sclerosis NAWM, axonal mGluR1 $\alpha$  IR was observed (arrows in D). (E) A confocal laser scanning microscope-generated image of mGluR1 $\alpha$ -positive axonal structures in white matter adjacent to affected areas. Sections were triple immunolabelled with antibodies to mGluR1 $\alpha$  (red colour), MOG (green colour) and non-phosphorylated neurofilaments (the SMI-32 antibody; blue colour). Arrows in E indicate the expression of mGluR1 $\alpha$  in an SMI-32-positive axon. Note also the presence of axons only immunoreactive for SMI-32 (arrowheads). (F) A chronic active lesion with axonal mGluR1 $\alpha$  IR. (G) mGluR1 $\alpha$  IR (red colour) in  $\beta$ -APP-positive axons (blue colour) in an active multiple sclerosis lesion (a higher magnification is shown in the insert). (H and I) mGluR1 $\alpha$  IR (red) in SMI-32-positive (blue) axons (arrows) (within an active multiple sclerosis lesion) which are either partially (H; MOG, green) or totally demyelinated (I). Strong mGluR1 $\alpha$  IR is observed in a reactive astrocyte in the border of a chronic active lesion (J). (K) A merged confocal laser scanning microscope-generated image of double-labelling with antibodies to mGluR1 $\alpha$  (red) and GFAP (green). The arrow indicates a GFAP-positive astrocyte expressing mGluR1 $\alpha$  (arrow). Note the presence of GFAP-positive astrocytes that lack mGluR1 $\alpha$  IR (arrowhead). (L) A merged confocal image showing co-localization of mGluR1 $\alpha$  (red) with vimentin (green) in a reactive glial cell (arrow), which is adjacent to an mGluR1 $\alpha$ -positive axonal structure (arrowheads). (M) A merged confocal image showing a section (from a chronic active lesion) triple immunolabelled with antibodies to mGluR1 $\alpha$  (red), MOG (green) and HLA-DP, DQ, DR (blue). Arrow points to mGluR1 $\alpha$  IR in a cell with an amoeboid, macrophage-like morphology. Other debris-laden macrophages present in the same field appear to be mGluR1 $\alpha$  negative (arrowheads). For A, B, C, D, F, bar = 50  $\mu$ m; J, bar = 25  $\mu$ m; E, G, H, I, K, L, M, bar = 10  $\mu$ m.



**Fig. 2** Distribution of mGluR5 and mGluR2/3 immunoreactivity (IR) in control and multiple sclerosis white matter. (A) mGluR5 and (B) mGluR2/3 IR in the subcortical white matter of normal control tissue with few stained astroglial cells (arrows). (C and D) Sections of a chronic active multiple sclerosis lesion stained for mGluR5 (C) and mGluR2/3 (D). Diffuse expression of both receptor subtypes is observed in virtually all reactive astrocytes. (E and F) Merged confocal images showing co-localization (yellow) of mGluR5 (red) with GFAP (green; E) and vimentin (green; F). (G) Co-localization (yellow) of mGluR2/3 (red) with vimentin (green). (H and I) Merged confocal images showing sections (from a chronic active lesion) triple immunolabelled with antibodies to mGluR5 (H; red) or mGluR2/3 (I; red), MOG (green) and HLA-DP, DQ, DR (blue). Arrows in H point to HLA-DP, DQ, DR-positive cells that were not stained with antibodies to mGluR5; the mGluR5-positive cells (arrowheads) resemble morphologically reactive astrocytes. (I) mGluR2/3 labelling in cells of the microglial/macrophage lineage (HLA-DP, DQ, DR-positive) with an amoeboid morphology. A–D bar = 50  $\mu$ m; E–I, bar = 10  $\mu$ m.

we performed an immunolabelling with Abs to HLA-DP, DQ, DR antigens on chronic active multiple sclerosis lesions containing microglial/macrophage lineage cells at different

stages of activation. mGluR2/3 IR, and to a lesser extent mGluR1 $\alpha$  IR, was detected in a population of cells with an amoeboid (macrophage-like) morphology, but not in cells





autopsy tissue with the relatively long post mortem time of the tissue, it was not possible to carry out an immunogold electron microscopic study to determine the precise localization of mGluR $\alpha$  within axons. Nonetheless, it is likely that the receptor protein molecules detected in axons in affected multiple sclerosis tissue were being transported from the cell soma to the axonal terminals. Previous electron microscopic studies on rapidly fixed biopsy control brain tissue have indicated that the IR of group I mGluRs is expressed primarily postsynaptically, although some labelling was also detected in axonal terminals (Ong *et al.*, 1998; Wang *et al.*, 2000; Hubert *et al.*, 2001). The most likely explanation for the presence of mGluR1 $\alpha$  axonal IR in multiple sclerosis tissue is accumulation due to a disturbance in axonal transport, as appears to be the case for a subunit of voltage-gated calcium channels (Kornek *et al.*, 2001). In our study, the axonal staining of mGluR $\alpha$  also shows a preferential localization in damaged axons, as evidenced by the presence of  $\beta$ -APP, which is a sensitive and early marker for impaired axonal transport (Ferguson *et al.*, 1997; Bitsch *et al.*, 2000; Kornek *et al.*, 2000; Kuhlmann *et al.*, 2002).

The data from several studies support a crucial contribution of mGluR1 $\alpha$  to synaptic transmission in both physiological and pathological conditions (for a review see Bordi and Ugolini, 1999). Changes in the expression of mGluR1 $\alpha$  protein have been reported in association with aberrant axonal organization, and in response to deafferentation (Casabona *et al.*, 1998; Blumcke *et al.*, 2000). The axonal mGluR1 $\alpha$  IR observed in our study (both in lesion areas and in NAWM) may reflect diffuse multiple sclerosis axonal pathology, involving both local axons (damaged due to processes of demyelination and inflammation) and axons localized distantly from the focus of demyelination (damaged due to deafferentation).

Whether the changes in mGluR1 $\alpha$  staining pattern described in this study contribute to the evolution of multiple sclerosis lesions is unclear, as both toxic and neuroprotective effects have been reported (reviewed in Bordi and Ugolini, 1999). Thus, upregulation of mGluR1 $\alpha$  in multiple sclerosis tissue could indicate an attempt to protect neurons from chronic excitotoxic injury. Recent data have suggested that group I mGluRs may limit oligodendrocyte progenitor cell degeneration during acute brain insults (Kelland and Toms, 2001).

Our study is a descriptive one and we were therefore not able to assess the functional consequences of the axonal expression of mGluR1 $\alpha$ . Further research in animal models of multiple sclerosis is clearly needed in order to elucidate the functional implication of changes in mGluR expression in the pathogenesis of the disorder.

### **Glial mGluR immunoreactivity in multiple sclerosis lesions**

Strong expression of both group I and group II mGluRs in glial cells with the morphology of reactive astrocytes was

observed in all of the active and chronic active multiple sclerosis lesions included in our study. This is essentially in agreement with previous reports showing that mGluR2/3 and mGluR5 are overexpressed in human reactive glial cells (Aronica *et al.*, 2001a, b). As previously shown, both membranous and cytoplasmic mGluR staining was observed in reactive astrocytes (Aronica *et al.*, 2000, 2001a, b, 2002). The presence of an intracellular pool of group I mGluR has also been shown by immunogold labelling electron microscopic studies in normal biopsy brain tissue (Hubert *et al.*, 2001). This may represent a reserve pool important for mGluR functions. Although hours are needed for new receptors to be synthesized and transported to the membrane, it appears that only minutes are required for intracellular receptors to be incorporated into the cell's membranes (Szekeres *et al.*, 1998).

Whereas virtually all reactive astrocytes displayed mGluR5 and mGluR2/3 IR, the expression of mGluR1 $\alpha$  was observed in only a subpopulation of reactive astrocytes. Whether this reflects different physiological functions for mGluR subtypes within the glial scar or reflects the presence of distinct subsets of astrocytes in multiple sclerosis lesions (Holley *et al.*, 2002) is still unclear. Activation of mGluR subtypes can regulate glial cell function and their interaction with neurons (Winder *et al.*, 1996; Cartmell and Schoepp, 2000). From our study using fixed material, it cannot be concluded whether the detected proteins were functionally active. However, cultures of rat and human astrocytes grown in conditions which result in the upregulation of the expression of mGluR mRNAs and proteins mimic the morphological changes of reactive glial cells observed *in vivo*, and this has been associated with increased receptor functions (Miller *et al.*, 1995b; Aronica *et al.*, 2002).

The potential role of astrocytes in the initiation, maintenance and regulation of immune responses in multiple sclerosis has been a matter of debate for many years (Lee and Brosnan, 1997; Aschner, 1998). Recent evidence indicates that astrocyte-mediated immune responses are regulated through activation of different mGluR subtypes (Bruno *et al.*, 1998; Besong *et al.*, 2002; E. Aronica, A.J. Rozemuller, J.A. Gorter, B. Yankaya and D. Froost, unpublished observations). Activation of group I and II mGluRs has also been shown to regulate glial cell proliferation differentially (Ciccarelli *et al.*, 1997; E. Aronica, A.J. Rozemuller, J.A. Gorter, B. Yankaya and D. Froost, unpublished observations) and glial glutamate transporter protein expression in rat and human astrocytes in culture (Aronica *et al.*, 2002).

Although cultured microglial cells have been shown to express mGluRs (Biber *et al.*, 1999; Taylor *et al.*, 2002), no information is available about the *in vivo* mGluR expression in human microglial/macrophage lineage cells under pathological conditions. In the present study, we provide evidence that mGluR2/3 and, to a lesser extent also mGluR1 $\alpha$ , are expressed in a subset of cells of the microglial/macrophage lineage in multiple sclerosis lesions. The expression of group II mGluRs in cells of the microglial/macrophage lineage in

multiple sclerosis tissue is in agreement with a recent study showing functional mGluR2/3 receptors in microglia grown *in vitro* (Taylor *et al.*, 2002).

Taken together, our findings suggest that changes in mGluR expression may be of relevance to glial function and glial–neuronal communication in multiple sclerosis tissue. Specific agonists and antagonists of mGluR subtypes are available and currently are used to evaluate the function of mGluRs in several pathological conditions (Bordi and Ugolini, 1999; Besong *et al.*, 2002). mGluR subtypes may thus represent an interesting pharmacological target for experimental therapeutic interventions in multiple sclerosis.

### Acknowledgements

We wish to thank The Netherlands Brain Bank (coordinator: Dr R. Ravid) for the collaboration in the collection of multiple sclerosis material, and Lisette Montagne, Elise van Haastert, Sandra Redeker for their excellent technical assistance and Dr W. Kamphorst and Dr P. J. W. Pouwels for their advice and helpful comments. J.J.G.G. (project 00-427MS) and G.W. (project 99-393) are supported by the ‘Stichting Vrienden MS Research’ Korschoten, The Netherlands. E.A. is supported by the Stichting AZUA-funds.

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Received February 3, 2003.

Revised March 10, 2003. Accepted March 25, 2003