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ORIGINAL ARTICLE

Enteric GFAP expression and phosphorylation in Parkinson's disease

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Abstract

Enteric glial cells (EGCs) are in many respects similar to astrocytes of the central nervous system and express similar proteins including glial fibrillary acidic protein (GFAP). Changes in GFAP expression and/or phosphorylation have been reported during brain damage or central nervous system degeneration. As in Parkinson's disease (PD) the enteric neurons accumulate α-synuclein, and thus are showing PD-specific pathological features, we undertook the present survey to study whether the enteric glia in PD become reactive by assessing the expression and phosphorylation levels of GFAP in colonic biopsies. Twenty-four PD, six progressive supranuclear palsy (PSP), six multiple system atrophy (MSA) patients, and 21 age-matched healthy controls were included. The expression levels and the phosphorylation state of GFAP were analyzed in colonic biopsies by western blot. Additional experiments were performed using real-time PCR for a more precise analysis of the GFAP isoforms expressed by EGCs. We showed that GFAPκ was the main isoform expressed in EGCs. As compared to control subjects, patients with PD, but not PSP and MSA, had significant higher GFAP expression levels in their colonic biopsies. The phosphorylation level of GFAP at serine 13 was significantly lower in PD patients compared to control subjects. By contrast, no change in GFAP phosphorylation was observed between PSP, MSA and controls. Our findings provide evidence that enteric glial reaction occurs in PD and further reinforce the role of the enteric nervous system in the initiation and/or the progression of the disease.

Keywords: enteric glial cells, enteric nervous system, glial fibrillary acidic protein, multiple system atrophy, Parkinson's disease, progressive supranuclear palsy.

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Glial fibrillary acidic protein (GFAP) is a major constituent of glial intermediary filaments that form the cytoskeleton of mature astrocytes. To date, nine splice variants of GFAP have been described in the human central nervous system (Kamphuis *et al.* 2014). GFAPα is the canonical isoform and most of the immunohistochemical studies on astroglia have used antibodies that do not discriminate between GFAP isoforms. The assembly of GFAP is controlled by its

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Abbreviations used: EGCs, enteric glial cells; ENS, enteric nervous system; GFAP, glial fibrillary acidic protein; MSA, multiple system atrophy; PAGE, polyacrylamide gel electrophoresis; PD, Parkinson's disease; PSP, progressive supranuclear palsy.

phosphorylation state, as its soluble phosphorylated pool is in dynamic equilibrium with the polymerized non-phosphorylated fraction of the protein (Inagaki *et al.* 1994). Several lines of evidence support a tight regulation of GFAP in neural development and also in the pathophysiology of several neurodegenerative disorders (Middeldorp and Hol 2011). GFAP gene mutations have indeed been associated with the fatal neurodegenerative condition Alexander disease (Yoshida and Nakagawa 2012). Furthermore, changes in GFAP expression and phosphorylation have been consistently reported in the central nervous system during neurodegenerative disorders such as Alzheimer's disease, frontotemporal dementia, and Parkinson's disease (PD) (Damier *et al.* 1993; Korolainen *et al.* 2005; Herskowitz *et al.* 2010; Kamphuis *et al.* 2014).

Astrocytes in the central nervous system are not the only cell type to express GFAP. In the early eighties, Jessen and Mirsky convincingly demonstrated that the glial cells in the enteric nervous system (ENS) were also immunoreactive for GFAP (Jessen and Mirsky 1980). This led to a reappraisal of the function and morphology of these glial cells, which were hitherto defined as Schwann cells. There is now a large body of evidence to support that the so-called enteric glial cells (EGCs) are in fact the digestive counterparts of central nervous system astrocytes (Gulbransen and Sharkey 2012). EGCs lie adjacent to the neurons in the enteric ganglia and envelop both the neuronal cell bodies and the axon bundles, an aspect that is highly reminiscent of the close relationship between astrocytes and neurons in the central nervous system (Jessen and Mirsky 1983). EGCs and astrocytes are also similar at the molecular and functional levels as they share electrophysiological and neuroprotective properties (Hanani 1993; Hanani *et al.* 2000; Abdo *et al.* 2010; Boesmans *et al.* 2013). In contrast to astrocytes, EGCs are readily accessible to biopsy and can therefore be analyzed in living patients (Lebouvier *et al.* 2010a; Neunlist *et al.* 2013). A routine colonic biopsy enables analysis of both mucosal and intraganglionic submucosal populations of EGCs and this approach has been used to demonstrate that GFAP is up-regulated in the gut of patients with inflammatory bowel disease (Boyen von *et al.* 2011).

It has become evident over the last 20 years that PD is not only a neurodegenerative brain condition but also a gut disorder (Cersosimo and Benarroch 2008; Derkinderen *et al.* 2011). Gastrointestinal symptoms are prominent non-motor manifestations of the disease (Edwards *et al.* 1993) and neuropathological studies showed the presence of Lewy pathology in the enteric neurons in the vast majority of patients (Beach *et al.* 2009). Our recent results showing an increase in total GFAP mRNA in the colon of PD patients suggest that enteric Lewy pathology in PD does not occur in isolation and may be accompanied by enteric glial reaction (Devos *et al.* 2013). In this study, we extended these preliminary results by determining whether this increase in GFAP expression is specific for some isoforms and whether associated post-translational modifications are involved.

Patients and methods

Subjects

A total of 57 subjects participated in this study: 24 PD, six progressive supranuclear palsy (PSP), and six multiple system atrophy (MSA) patients as well as 21 healthy controls. PD patients aged 40–75 years were recruited from the movement disorder clinic at Nantes University Hospital, France. Diagnosis was made according to criteria provided by the United Kingdom Parkinson's Disease Survey Brain Bank (Hughes *et al.* 2002). PSP and MSA patients fulfilled the diagnostic criteria for possible or probable PSP (Litvan *et al.* 1996) and MSA (Gilman *et al.* 2008), respectively. Control subjects were healthy subjects who had a normal colonoscopy performed for colorectal cancer screening. All controls subjects underwent a detailed neurological examination to rule out PD symptoms and cognitive deficiency. The study protocol was approved by the local Committee on Ethics and Human Research (*Comité de Protection des Personnes Ouest VI*), conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and registered on ClinicalTrials.gov (identifier NCT00 491062 and NCT01353183). Written informed consent was obtained from each patient and from each normal volunteer.

Endoscopic procedure and colonic biopsies

Four biopsies were taken in the sigmoid/descending colon during the course of a rectosigmoidoscopy for PD, PSP, and MSA patients and during a colonoscopy for control subjects. A follow-up call was scheduled 15 days after the endoscopic procedure. Biopsies were performed using standard biopsy forceps without needle (FB210K; Olympus co., Tokyo, Japan). Two biopsies were stored at −80°C in lysis buffer RA1 (Macherey-Nagel, Hoerdt, France) for further analysis by real-time PCR and immunoblotting. The two remaining biopsies were snap frozen in liquid nitrogen at the time of collection and kept at −80°C.

Rat enteroglial cell line and treatment with serine/threonine phosphatases inhibitors

Enteric glial cell line was generated and cultured as previously described (Van Landeghem *et al.* 2011). At confluence, cells were treated with a cocktail of three phosphatase inhibitors for broad-spectrum inhibition of serine/threonine phosphatases including 1 μM okadaic acid, 5 μM ciclosporine A, and 6.75 μM sanguinarine (Sigma, Saint Quentin Fallavier, France) for 3 h or with vehicle (100 mM NaCl, 2 mM EDTA, 50 mM Tris-Cl, pH 7.4, and 50 mM NaF).

Human brain sample

A coronal frozen section of a human brain devoid of neurodegenerative pathological changes passing through the head of caudate nucleus (approximately 10 mm thick) was kindly provided by Pr Charles Duyckaerts, CRICM, Salpêtrière, Paris, France. Samples of frontal cortex and subventricular zone (SVZ) were taken and lysed in NETF (100 mM NaCl, 2 mM EDTA, 50 mM Tris-Cl, pH 7.4, and 50 mM NaF) buffer (protein concentration: 12 μg/μL for undiluted cortex sample and 2 μg/μL for undiluted SVZ sample) for western

blot analyses (see below). Because GFAPδ is mainly observed in the adult SVZ, a lysate of this brain region at a 1 : 10 dilution was used for the evaluation of the expression of this specific isoform (Roelofs *et al.* 2005). Lysate of frontal cortex either diluted at 1 : 10 or 1 : 1000 was used for the evaluation of the expression of all others GFAP isoforms.

Western blot

Following RNA isolation, total proteins from the two-pooled biopsies were precipitated and prepared for polyacrylamide gel electrophoresis (PAGE) using protein precipitator and resuspension buffer [Protein solving buffer and TCEP (tris(2-carboxyethyl) phosphine) reducing agent, PSB/TCEP] from NucleoSpin Triprep Kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. For additional experiments on GFAP isoforms and phosphorylation, the two remaining biopsies that were dry frozen and stored at −80°C were lysed in NETF buffer containing 1% (v/v) IGEPAL® CA-630, 2 mM orthovanadate, phosphatase inhibitor cocktail II (Roche, Neuilly sur Seine, France) and a protease inhibitors cocktail (Roche) using the 'Precellys 24' tissue homogenizer (Bertin technologies, St Quentin-en-Yvelines, France) and followed by sonication with 'vibracell 75 186' device (Sonics, Newton, CT, USA). Human brain samples and EGCs were processed in the same way. Total protein was quantified using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Cillebon sur Yvette, France) for samples prepared with PSB/TCEP and bicinchoninic acid protein assay kit (Pierce Thermo Scientific, Illkirch, France) for samples lysed in NETF buffer. Equal amounts of lysate were separated using the Invitrogen NuPage Novex Bis Tris MiniGels™ together with the 2-(N-morpholino)ethanesulfonic acid/sodium dodecyl sulfate (MES-SDS) running buffer before electrophoretic transfer to nitrocellulose membranes with the iBlot™ Dry Blotting System also from Invitrogen (Invitrogen, Cergy-Pontoise, France). In some experiments, the MES-SDS buffer was replaced by the 3-morpholinopropane-1-sulfonic acid/sodium dodecyl sulfate (MOPS-SDS) running buffer. Membranes were blocked for 1 h at 25°C in Tris-buffered saline (TBS) (150 mM NaCl, 15 mM Tris, 4.6 mM Tris Base, pH 7.4) with 5% non-fat dry milk and incubated overnight at 4°C with the primary antibodies. Details on the primary antibodies against GFAP are summarized in Table 1; mouse monoclonal anti-β-actin antibody was from Sigma and used at 1 : 10 000; rabbit anti-extracellular signal-regulated kinases was from Cell Signaling (Ozyme, Saint Quentin en Yvelines, France) and used at 1 : 1000. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit, anti-mouse antibodies (Amersham, Les Ulis, France; diluted 1 : 5000) or anti-goat antibody (Santa Cruz Biotechnologies, Cliniscience, Nanterre, France; diluted 1 : 5000) and visualized by enhanced chemiluminescent detection (ECLPrime, Amersham). When necessary, membranes were stripped for 15 min in Reblot buffer Strong™ (Millipore, Molsheim, France) followed by extensive washing in TBS before reblocking for 30 min in TBS with 5% non-fat dry milk and reprobing. The relevant immunoreactive bands were quantified with laser-scanning densitometry and analyzed with NIH Image J software. To allow comparison between different autoradiographic films, the density of the bands was expressed as a percentage of the average of controls. The value of GFAP was normalized to the amount of beta-actin or extracellular signal-regulated kinases (ERKs), for comparison between biopsies and between biopsies and brain samples, respectively, in the same sample and expressed as a percentage of the average of controls. Phospho-GFAP immunoreactivity was normalized to GFAP immunoreactivity.

Membrane dephosphorylation

The specificity of the pSer13 antibodies (Table 1) was tested using western blotting and dephosphorylation treatment. Protein homogenates were electrophoresed, transferred to nitrocellulose membrane, and blocked for 1 h in TBS with 5% non-fat dry milk as performed above. The membrane to be dephosphorylated was placed in buffer (50 mM sodium acetate at pH 5.5 and 0.1% Tween 20) with 0.5 mg/mL acid phosphatase (Sigma) and incubated overnight at 37°C with gentle agitation. The control membrane was processed similarly but without acid phosphatase. Western blotting and stripping of membranes were performed as above.

Real-time PCR analysis

Total RNA (1.0 μg) was DNase I treated and used as a template to generate cDNA following the manufacturer's instructions (QuantiTect Reverse Transcription Kit-Qiagen, Courtaboeuf, France) with a blend of oligo-dT and random hexamer primers. The reverse transcriptase reaction was incubated at 42°C for 30 min. The resulting cDNA was diluted 1 : 20 and served as a template in real-time qPCR assays (SYBR-Green PCR Master Mix; Applied Biosystems, Courtaboeuf, France). Primers were generated for the specific GFAP isoforms and tested for efficiency. The PCR signal was normalized against a set of reference genes to control for variability in the amount and quality of the RNA. Primer specificity and sensitivity are tested on dilution series of cloned GFAP isoforms to prevent unwanted cross-amplification of GFAPα, the most abundant transcript; details and primer sequences were described previously (Kamphuis *et al.* 2014).

Table 1 Human GFAP isoform-specific antibodies used in the present study

Name	Specificity	Epitope	Source and dilution
PanGFAP	α, δ, κ, Δ135, Δex6,Δ164	Full-length GFAP cow	Dako, Les Ulis, France, rabbit polyclonal Z0334 (1 : 2000)
GFAPmono	α, δ, κ, Δ135	ITIPVQTFSNLQIR	Sigma, mouse monoclonal GA5 (1 : 1000)
GFAP N-term	α, δ, κ, Δ135	MERRRITSAARRSYVSSGEMMV	SCBT, mouse monoclonal F-2 (1 : 500)
GFAP C-term	α, Δ135	EMRDGEVIKESKQEHKDVM	SCBT, goat polyclonal C-19 (1 : 500)
GFAP δ	δ	QAHQIVNGTPPARG	Millipore, rabbit polyclonal (1 : 500)
GFAP κ	κ	SLGAFVTLQRS	NIN, rabbit polyclonal (1 : 500)
pSer13	℗Ser13	aa sequence around ℗Ser13	SCBT, rabbit polyclonal (1 : 1000)

Statistics

All data are given as the mean ± SEM. For comparisons of means between groups, a Mann–Whitney test was performed. Differences were deemed statistically significant if $p < 0.05$.

Results

Clinical features

Clinical features of the study population are shown in Table 2. Age and sex did not differ significantly between patients and control subjects. No complications occurred in the 57 patients included in this study, either during or after the endoscopic procedure.

Enteric GFAP expression is increased in PD but not in related disorders

In a first set of experiments, we evaluated the expression levels of GFAP in colonic biopsies by western blot with an antibody that recognizes the large majority of known isoforms and truncated forms of the protein (PanGFAP antibody, Table 1) (Middeldorp and Hol 2011; Kamphuis *et al.* 2012, 2014). The amounts of GFAP protein in colonic biopsies from PD patients were compared to samples from healthy subjects and patients with PSP and MSA, two atypical parkinsonian syndromes in which the ENS, in contrast with PD, is spared by the pathological process (Wakabayashi *et al.* 2010; Pouclet *et al.* 2012).

Detection of GFAP on western blots of protein samples from colonic biopsies with the PanGFAP antibody revealed one prominent and two weaker bands, migrating at 55, 50, and 45 kDa, respectively (Fig. 1a). When the density of all three GFAP-immunoreactive bands was assessed, a significant 1.2 fold increase in GFAP expression was observed in biopsies from PD patients as compared with controls (Fig. 1b). By contrast, the expression levels of GFAP in biopsies from PSP and MSA patients were significantly lower than in healthy controls (Fig. 1b). A separate analysis of each of the three GFAP-immunoreactive bands showed that the density of the 55-kDa band was significantly lower in patients with atypical parkinsonism compared with control subjects (Fig. 1c). A significant 1.6 fold increase in the density of the 50-kDa band was observed in PD patients when compared with controls (Fig. 1d), while the density of the 45-kDa band was not different between controls and patients (Fig. 1e).

GFAPκ is the main isoform expressed in enteric glia

To date, nine splice variants of GFAP have been described in the human central nervous system (Middeldorp and Hol 2011; Kamphuis *et al.* 2014). GFAPα, δ, and κ encode long isoforms of the protein of 432, 431, and 438 amino acids in length, respectively, that migrate together on PAGE at approximately 55 kDa (Fig. 2 and Table 3) (Middeldorp and Hol 2011; Kamphuis *et al.* 2014). GFAP Δ135, ΔEx6, and Δ164 encode shorter isoforms of the protein of 387, 366, and 347 amino acids, respectively, with a faster migration profile on PAGE ranging between 40 and 45 kDa (Hol *et al.* 2003). All these isoforms are recognized by the PanGFAP antibody (Fig. 2) (Kamphuis *et al.* 2014). Regarding the three remaining known isoforms GFAPβ, γ, and ζ, their detection at the protein level by PanGFAP or other anti-GFAP antibodies has never been tested (Zelenika *et al.* 1995; Condorelli *et al.* 1999). This suggests that the major 55-kDa migrating band we observed in Fig. 1 contains at least GFAPα, δ, and/or κ, whereas the two fastest migrating band at 50 and 45 kDa represent the alternatively spliced Δ135, ΔEx6, Δ164 isoforms and/or truncated forms of GFAP that can be generated by N-terminal post-translational truncation (Lee *et al.* 2000; Zoltewicz *et al.* 2012).

To better identify the GFAP isoforms expressed by enteric glia, the immunoreactivity of five additional antibodies was analyzed in both colonic biopsies and human brain extracts using an adapted western blotting approach. Western blot experiments were performed using the MOPS-SDS running buffer as this buffer allowed a better separation of the GFAP isoforms when compared to the MES-SDS running buffer that was used in Fig. 1 (Fig. 3a). The first antibody we used (GFAPmono), which is specific for GFAPα, δ, κ, and Δ135 (Table 1 and Fig. 2) (Middeldorp and Hol 2011; Kamphuis *et al.* 2014) detected one major band along with several faster migrating bands in human frontal cortex lysate (Fig. 3b and Figure S1). The pattern of immunoreactivity was different in colonic biopsies with one single major band migrating at 55 kDa in most samples (Fig. 3b). Faint faster migrating bands were visible in some biopsies lysates (see lane 1, Fig. 3b). The second antibody we used (GFAP C-term) is specific for the carboxy-terminal tail of GFAPα and the alternatively spliced variant GFAP Δ135 (Table 1 and Fig. 2) (Middeldorp and Hol 2011; Kamphuis *et al.* 2014). This antibody detected one band at 55 kDa when the human frontal cortex lysate was diluted at 1 : 1000 (Fig. 3b) and

Table 2 Demographic data of control subjects and patients

	Controls (n = 21)	PD (n = 24)	PSP (n = 6)	MSA (n = 6)
Age, years	63.6 ± 2 (39–75)	61.1 ± 1.5 (44–71)	71 ± 2 (63–75)	61 ± 4.1 (51–71)
Gender, M/F	11/10	13/11	3/3	3/3

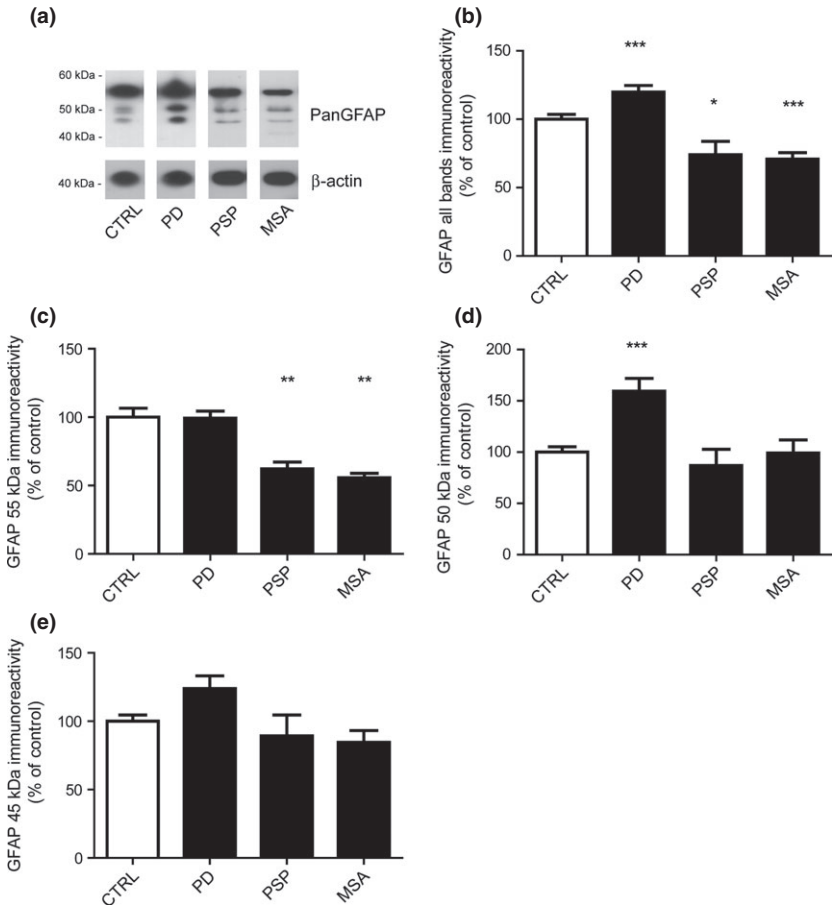

Fig. 1 Glial fibrillary acidic protein (GFAP) expression in colonic biopsies from patients with Parkinson's disease (PD), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), and control subjects (CTRL). (a) Biopsies lysates (10 μg of protein per sample) were subjected to immunoblot analysis using an antibody recognizing most known GFAP isoforms (GFAP, PanGFAP in Table 1) and beta-actin. The PanGFAP antibody detected three main bands in lysates of colonic biopsies, one major and two fainter bands, migrating at 55, 50, and 45 kDa, respectively. (b) Global quantification of all GFAP-immunoreactive bands. The optical densities of all three GFAP-immunoreactive bands were measured, normalized to the optical densities of beta-actin immunoreactive bands in the same samples, expressed as percentages of controls and added. Data correspond to mean ± SEM of 21 samples for control subjects (CTRL), 24 samples for PD patients and six samples for PSP and MSA. Patients versus control, $*p < 0.05$ and $***p < 0.001$. (c, d, e) Individual quantification of the three GFAP-immunoreactive bands at 55, 50, and 45 kDa. The optical densities of each GFAP-immunoreactive band were measured, normalized to the optical densities of beta-actin immunoreactive bands in the same samples, and expressed as percentages of controls. Data correspond to mean ± SEM of 21 samples for CTRL, 24 samples for PD patients and six samples for PSP and MSA. Patients versus control, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

two main bands migrating at 55 and 45 kDa when the 1 : 10 diluted lysate was used (Figure S1). In colonic biopsies, this antibody either detected a faint band at 55 kDa (Fig. 3b) or no specific band (Fig. 1). The third antibody (GFAP N-term), which recognizes intact GFAPα, δ, κ, and Δ135 but not their amino-terminal cleavage products (Table 1 and Fig. 2) detected one band migrating at 55 kDa in human frontal cortex lysate diluted at 1 : 1000 (Fig. 3b) and two bands, one major and one minor migrating at 55 and 50 kDa, respectively, in the more concentrated brain lysate (Figure S1). A single major 55-kDa band was observed when this antibody was used in colonic biopsies (Fig. 3b and Figure S1). An antibody specific for human GFAPδ (Table 1 and Fig. 2) that does not cross-react with other splice variants (Roelofs *et al.* 2005) failed to detect any specific band at the expected molecular weight in colonic biopsies (Fig. 3b), while it recognized a band migrating at the expected size in SVZ lysate (Fig. 3b). Finally, an antibody raised against a specific sequence of GFAPκ (Table 1) (Kamphuis *et al.* 2014) detected one major strong band migrating at 55 kDa in biopsies and a fainter band of approximately the same size in frontal cortex lysate (Fig. 3b). An additional faster migrating band was also observed in some samples (Fig. 3b). Taken together these

GFAP Alpha 432 aa, 49.9 kDa

```
1 merrritsaa rrsyvssgem mvgglapgrr lgpgtrlsla rmppplptrv dfslagalna
      61 gfketraser aemmelndrf asyiekvrfl eqqnkalaae lnqlrakept kladvyqael
     121 relrlrldql tansarleve rdnlaqdlat vrqklqdetn lrleaennla ayrqeadeat
     181 larldlerki esleeeirfl rkiheeevre lqeqlarqqv hveldvakpd ltaalkeirt
     241 qyeamassnm heaeewyrsk fadltdaaar naellrqakh eandyrrqlq sltcdleslr
     301 gtneslerqm reqeerhvre aasyqealar leeegqslkd emarhlqeyq dllnvklald
     361 ieiatyrkll egeenritip vqtfsnlqir etsldtksvs eghlkrnivv ktvemrdgev
     421 ikeskqehkd vm
```

GFAP Delta 431 aa, 49.5 kDa

```
1 merrritsaa rrsyvssgem mvgglapgrr lgpgtrlsla rmppplptrv dfslagalna
      61 gfketraser aemmelndrf asyiekvrfl eqqnkalaae lnqlrakept kladvyqael
     121 relrlrldql tansarleve rdnlaqdlat vrqklqdetn lrleaennla ayrqeadeat
     181 larldlerki esleeeirfl rkiheeevre lqeqlarqqv hveldvakpd ltaalkeirt
     241 qyeamassnm heaeewyrsk fadltdaaar naellrqakh eandyrrqlq sltcdleslr
     301 gtneslerqm reqeerhvre aasyqealar leeegqslkd emarhlqeyq dllnvklald
     361 ieiatyrkll egeenritip vqtfsnlqir ggkstkdgen hkvtrylksl tirvipiqah
     421 qivngtppar g
```

GFAP Kappa 438 aa, 50.3 kDa

```
1 merrritsaa rrsyvssgem mvgglapgrr lgpgtrlsla rmppplptrv dfslagalna
      61 gfketraser aemmelndrf asyiekvrfl eqqnkalaae lnqlrakept kladvyqael
     121 relrlrldql tansarleve rdnlaqdlat vrqklqdetn lrleaennla ayrqeadeat
     181 larldlerki esleeeirfl rkiheeevre lqeqlarqqv hveldvakpd ltaalkeirt
     241 qyeamassnm heaeewyrsk fadltdaaar naellrqakh eandyrrqlq sltcdleslr
     301 gtneslerqm reqeerhvre aasyqealar leeegqslkd emarhlqeyq dllnvklald
     361 ieiatyrkll egeenritip vqtfsnlqir gqysrasweg hwspapssra crllqtgted
     421 qgkgiqlslg afvtlqrs
```

GFAP Δ135 387 aa, 44.5 kDa

```
1 merrritsaa rrsyvssgem mvgglapgrr lgpgtrlsla rmppplptrv dfslagalna
      61 gfketraser aemmelndrf asyiekvrfl eqqnkalaae lnqlrakept kladvyqael
     121 relrlrldql tansarleve rdnlaqdlat vrqklqdetn lrleaennla ayrqeadeat
     181 larldlerki esleeeirfl rkiheeevre lqeqlarqqv hveldvakpd ltaalkeirt
     241 qyeamassnm heaeewyrsk fadltdaaar naellrqakh eandyrrqlq sltcdleslr
     301 gtnyqdllnv klaldieiat yrkllegeen ritipvqtfs nlqiretsld tksvseghlk
     361 rnivvktvem rdgevikesk qehkdvm
```

Fig. 2 Epitopes recognized by the different anti-glial fibrillary acidic protein (GFAP) antibodies in the four main human GFAP isoforms. GFAPmono (single underline); GFAP C-term (double underline); GFAP δ (dashed underline); GFAP N-term (thick underline); GFAP κ (bold wavy underline). NCBI reference sequence: GFAP α: NP_002046.1; GFAP δ: NP_001124491.1; GFAP κ: NP_001229305.1. The expected molecular weights are indicated.

Table 3 Results of quantitative polymerase chain reaction assays

	C	C	C	PD	PD	PD	PD	PD	PD	Mean ± SEM
κ/α	0.28	2.40	0.78	1.28	1.32	0.18	0.13	4.94	3.83	1.68 ± 0.57
β/α	0.018	–	0.026	0.036	–	0.001	–	0.029	–	0.022 ± 0.006

Data are presented as ratio of GFAPκ and GFAPβ transcripts to GFAPα transcript.

results show that enteric glia does not express GFAPδ and Δ135, and that GFAPα is only a minor component of the main 55 kDa band detected by PanGFAP. Furthermore, the results obtained with GFAPκ and GFAP N-term antibodies strongly suggest that GFAPκ accounts for most of the GFAP immunoreactivity observed at 55 kDa and that the fastest migrating bands observed in PanGFAP immunoblots represent truncated products of this isoform.

To further refine the GFAP isoforms that are expressed by EGCs, PCR analyses were performed in biopsies from three control subjects and six PD patients. Transcripts for GFAPκ and α were the only ones to be consistently detected in all nine samples with the κ transcripts being 1.7-fold more abundant than the α transcript (Table 3). GFAPβ transcript was detected in five of the nine samples with an average β/α ratio of 2% (Table 3). All other isoforms transcripts, including GFAPδ and Δ135, were not or barely detectable. As a whole, our results obtained at the transcript and protein levels are consistent and demonstrate that GFAPκ is the main isoform expressed in enteric glia.

Enteric GFAP phosphorylation at serine 13 is decreased in PD

GFAP can be phosphorylated at multiple sites in its amino-terminal domain, including threonine 7, serine 8, 13, 17, and 34 (Inagaki *et al.* 1994). Among these sites, serine 13 has recently received much attention as it has been shown to be regulated during central nervous system neurodegeneration (Herskowitz *et al.* 2010). We have therefore studied the regulation of GFAP phosphorylation at this residue in the ENS using a rabbit polyclonal phospho-specific antibody (pSer13, Table 1). As a first step, we have validated the specificity of this antibody in enteric glia by treating rat enteroglial cells with a combination of serine/threonine phosphatase inhibitors (okadaic acid, ciclosporin A, and sanguinarine) and by incubating nitrocellulose membranes

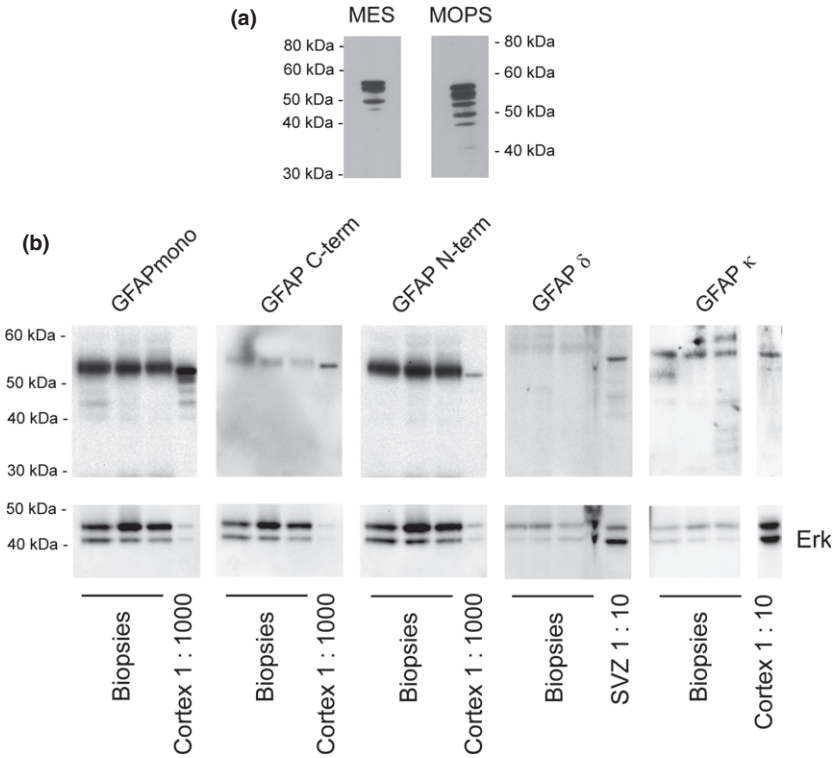

Fig. 3 Glial fibrillary acidic protein (GFAP) isoforms expressed by enteric glia. (a) Comparison of immunoblot profiles between MES-SDS and MOPS-SDS running buffers. A colonic biopsy from a control subject was subjected to PanGFAP immunoblot analysis using either MES-SDS running buffer (MES) and MOPS-SDS running buffer (MOPS). (b) Colonic biopsies and brain samples lysates from healthy subjects were subjected to immunoblot analysis using the MOPS-SDS running buffers and various antibodies against specific isoforms of GFAP: GFAPmono specific for GFAPα, δ, κ, and Δ135; GFAP C-term specific for the carboxy-terminal tail of GFAPα and of the alternatively spliced variant GFAP Δ135; GFAP N-term, which recognizes intact GFAPα, δ, κ, and Δ135 but not their amino-terminal cleavage products; isoform-specific anti-GFAPδ and anti-GFAPκ. The human frontal cortex lysate (Cortex) was diluted at 1 : 1000 for experiments with GFAPmono, GFAP C-term, and GFAP N-term, as these antibodies detect GFAPα, the most abundant GFAP isoform in brain. Human frontal cortex and subventricular zone (SVZ) lysates were both diluted at 1 : 10 for the evaluation of the GFAPκ and GFAPδ isoforms, respectively. Extracellular signal-regulated kinases (ERK) immunoblot was used as a loading control between biopsies and brain samples.

with acid phosphatase. pSer13 detected one major band migrating at 55 kDa in rat enteroglial cells only in the presence of phosphatase inhibitors (Fig. 4a). No immunoreactivity for pSer13 was observed if the membranes were dephosphorylated, while immunoblotting for PanGFAP revealed that regular GFAP was detected in both the normal and the dephosphorylated membrane (Fig. 4a). In colonic biopsies, pSer13 detected two bands, one major and one minor at 55 and 50 kDa, respectively, that comigrated with the two matching bands labeled by PanGFAP (Fig. 4b). Although highly variable between individuals, quantification of the X-ray films revealed that the expression levels of GFAP phosphorylated at serine 13 were significantly lower in PD patients as compared with control subjects (Fig. 4b, c and Figure S2). By contrast, no significant changes in pSer13 immunoreactivity were observed between PSP and MSA patients when compared with controls (Fig. 4b, c). Because the RA1 buffer that was used for storage and lysis of biopsies does not contain any phosphatase inhibitors, additional experiments were performed to show that our results were not the mere consequence of sample dephosphorylation. To this end, snap-frozen biopsies were lysed at 4°C in the presence of the tyrosine phosphatase inhibitor sodium orthovanadate and serine/threonine phosphatase inhibitors before being processed for western blotting. A decrease in pSer13 immunoreactivity was still observed in colonic biopsies from PD patients when this lysis method was used, confirming that our results were not because of artifactual dephosphorylation during sample preparation (Figure S3).

Discussion

Altogether, our previous results (Devos *et al.* 2013) and this study show that the expression of GFAP is increased at both mRNA and protein levels in mucosal and submucosal EGCs in PD. In analogy with the central nervous system, it has been proposed that GFAP up-regulation in the gut is induced by the activation of EGCs, a phenomenon known as reactive

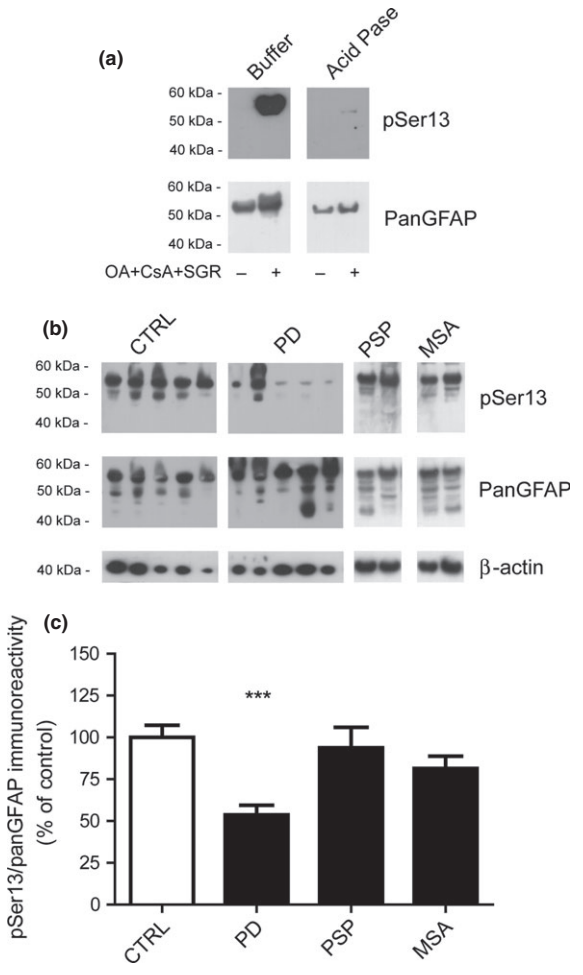

Fig. 4 Glial fibrillary acidic protein (GFAP) phosphorylation at serine 13 in colonic biopsies from Parkinson's disease (PD), multiple system atrophy (MSA) and progressive supranuclear palsy (PSP) patients and in control subjects. (a) The specificity of the antibody against the phosphorylated form of GFAP phosphorylated at serine 13 (pSer13) was assessed in enteroglial cells treated or not with a cocktail of three phosphatase inhibitors including 1 μM okadaic acid, 5 μM ciclosporine A, and 6.75 μM sanguinarine (OA+CsA+SGR) for 3 h or with vehicle. Fifteen μg of cell lysates were subjected to immunoblot analysis, with the nitrocellulose membrane being treated with acid phosphatase (acidPase) or not (buffer) before incubation with pSer13 antibodies. (b) Biopsies lysates (15 μg of protein per sample) were subjected to immunoblot analysis using pSer13 antibody and reprobed with PanGFAP and beta-actin antibodies. The GFAP-Phospho-Ser13 antibody detects two bands at 55 and 50 kDa that comigrate with the two corresponding bands detected by PanGFAP antibody, either in biopsies from controls (CTRL), PD, PSP or MSA. (c) Quantification of the two immunoreactive bands at 55 and 50 kDa detected by the pSer13 antibody. The optical densities of the two pSer13-immunoreactive bands were measured, normalized to the optical densities of PanGFAP-immunoreactive bands in the same samples, expressed as percentages of controls and added. Data correspond to mean ± SEM of 12 samples for CTRL, 19 samples for PD patients, 6 samples for MSA, and 5 samples for PSP patients (patients vs. control, ***$p < 0.001$).

gliosis. In the past few years, several studies have demonstrated that EGCs are critically involved in maintaining gut homeostasis (Neunlist *et al.* 2013) and especially in regulating gut inflammation (Cabarrocas *et al.* 2003; Ruhl 2005). Reactive gliosis as well as morphologically altered EGCs have been reported in the gut of patients with inflammatory bowel disease (Cornet *et al.* 2001; Boyen von *et al.* 2011) and studies in transgenic animals have showed that enteric glia ablation resulted in severe gut inflammation (Bush *et al.* 1998; Cornet *et al.* 2001; Aube *et al.* 2006). Further supporting the link between EGCs and inflammation, *in vitro* experiments obtained in cultured EGC showed that pro-inflammatory cytokines such as tumor necrosis alpha increase the expression levels of GFAP (Boyen von *et al.* 2004) and that once reactive, enteric glia is capable of secreting interleukin-6 (Ruhl *et al.* 2001). Regarding PD, we have recently shown that the expression levels of the main pro-inflammatory cytokines were increased in the colonic biopsies from PD patients and correlated with the expression of GFAP mRNA (Devos *et al.* 2013). By demonstrating the occurrence of glial reaction in the gut of PD patients, our results strongly support the assumption that PD is not restricted to the brain but is rather a systemic disorder that affects the peripheral autonomic networks and in particular the ENS (Braak and Del Tredici 2008; Beach *et al.* 2009). The data also reinforce the role of peripheral inflammation and associated glial reaction in the initiation and progression of the disease (Lema Tomé *et al.* 2013).

Various degrees of astrocytic reaction have been reported in the brain of parkinsonian syndromes, including PD, PSP, and MSA. In PD, reactive astrogliosis is usually mild, but abnormal synuclein deposition occurs in astrocytes, whereas MSA and PSP cases show marked astrocytic reaction, which is thought to contribute to neurodegeneration (Song *et al.* 2009). We show in the present report that, by contrast to PD, the levels of GFAP in colonic biopsies from PSP and MSA patients are either comparable or lower to the control subjects. This lack of increase in GFAP expression in the gut of MSA and PSP patients is a strong argument against the occurrence of enteric reactive gliosis and strongly suggests that the pathology in PSP and MSA is limited to the central nervous system. This is further supported by a small set of studies that showed the absence or paucity of pathologic lesions in the peripheral nervous systems in these two disorders (Wakabayashi *et al.* 2010; Pouclet *et al.* 2012).

Regarding the GFAP isoforms, our results were consistent between real-time PCR and western blot analyses and showed that GFAPκ, which is the most recently discovered GFAP isoform (Blechingberg *et al.* 2007), is by far the major isoform expressed by EGCs obtained from gastrointestinal biopsies. This stands in sharp contrast with the data that were recently obtained in human brain showing that the median expression level of GFAPκ transcript was 1.1% when the

level of GFAPα was set at 100% (Kamphuis *et al.* 2014). The GFAPκ protein has a C-terminal tail that is different from the C-terminal tails of the GFAPα and GFAPδ isoforms and it has been suggested that these differences may have a physiological consequence as GFAPκ, in contrast with GFAPα, has a low propensity to form homomeric intermediate filaments (Blechingberg *et al.* 2007). Whether the high expression level of GFAPκ in the gastrointestinal tract has an impact on the physiology of EGCs still needs to be studied. Furthermore, it remains to be determined if the EGCs from the myenteric plexus, which are not accessible to routine gastrointestinal biopsies, also preferentially express GFAPκ like their mucosal and submucosal counterparts.

The phosphorylated residues located at the amino terminus of GFAP, including threonine 7, serine 8, 13, 17, and 34, are involved in the regulation of the protein self-assembly (Inagaki *et al.* 1994). Phosphorylation of GFAP at its amino-terminus residues causes disassembly of the intermediate filaments and conversely its dephosphorylation restores its potential to assemble (Takemura *et al.* 2002). As the integrity of the cytoskeleton is essential for normal astrocyte function, it has been suggested that GFAP phosphorylation may be an important regulatory mechanism in central nervous system disorders. In spite of this, there are very few studies that have examined the phosphorylation state of GFAP in pathological conditions. Using two-dimensional immunoblotting, Korolainen and collaborators convincingly showed that the total amount of phosphorylated GFAP was increased in Alzheimer's diseases brains (Korolainen *et al.* 2005). Only two reports used a phospho-specific antibody to examine GFAP phosphorylation in brain neurodegeneration or insults. Using a phosphoproteomic analysis, Herskowitz *et al.* showed that the phosphorylation of GFAP at serine 13 was increased in the brain of individuals with frontotemporal lobar degeneration, a result that was validated by immunoblot with a phospho-specific antibody for GFAP phosphorylated at serine 13 (Herskowitz *et al.* 2010). Recently, Sullivan *et al.* (2012) demonstrated that the occurrence of hypoxia in pig brains was associated with increased amount of phosphorylation of GFAP at serine 13. Our findings showing GFAP hypophosphorylation at serine 13 in enteric glia during PD are thus apparently at odds with previous observations in brain damage. Nevertheless, given the role of GFAP phosphorylation in the plasticity of glia cytoskeleton, it could be argued that glial cells respond differently depending on the neurodegenerative process and that, by contrast to Alzheimer's disease and frontotemporal dementia, reactive gliosis in PD is associated with a drop in GFAP phosphorylation. In this respect, it is worth noting that the phosphorylation state of GFAP has hitherto not been investigated in the brain of PD patients. As a logical follow-up of our study, it would be logical to study whether GFAP is also hypophosphorylated in brain astrocytes during PD.

There is increasing interest in bidirectional signaling between the gut microbiota and brain and the potential impact of this communication on the development of psychiatric and neurological disorders, leading to the concept of a microbiota–gut–brain axis (Forsythe *et al.* 2012). Although the precise mechanisms through which signals from gut bacteria are communicated to the brain are still largely unknown, evidence obtained from vagotomy experiments point toward a key role for the vagus nerve in the interplay between the microbiota and the brain (Forsythe *et al.* 2012). In this context, the enteric neurons, which are embedded in the wall of the gastrointestinal tract, show some unique features that make them prime candidates to act as a first relay between gut microbiota and the brain as: (i) some of them, located in the submucosal plexus send axons to the gut mucosa that are only micrometers away from the gut lumen and thus from the gut flora, (ii) their neurochemical phenotype and electrophysiological properties can be modulated by changes in the composition of gut microbiota (Kunze *et al.* 2009), and (iii) they synapse with both afferent and efferent vagal neurons (Walter *et al.* 2009). With regard to PD, Braak suggested that the involvement of the enteric neurons by Lewy pathology was an early event in the development of the disease. This led to the assumption, the so-called Braak's hypothesis, that PD pathology may in fact begin in the gastrointestinal tract further spreading to the central nervous system via the vagal pre-ganglionic innervation of the gut and thus following the brain–gut axis (Braak *et al.* 2006). Our results, showing significant changes in EGC during PD, further reinforce a possible role of the enteric nervous system in the initiation or the progression of the disease. Further work will be needed to determine whether changes in gut microbiota occur in PD and whether these changes are capable of modifying enteric neurons and EGC.

Despite technological advances in the field of molecular genetics and in *in vivo* imaging, no fully validated biomarker for PD is available yet (Marek *et al.* 2008) and there is still a need for new biomarkers that will complement the ones already available The observations that demonstrated that Lewy pathology is not limited to the central nervous system but also involves peripheral tissues accessible to biopsies including skin, salivary glands, and gut, provide new opportunities to develop original histopathological markers of the disease that will directly assess the pathological process *in vivo* (Lebouvier *et al.* 2010c). Remarkably, by contrast to skin and salivary glands, a gut biopsy does not only contain post-ganglionic neuronal processes but a dense network of neurons and EGCs (Lebouvier *et al.* 2010a,b). By showing differences in the expression and phosphorylation of GFAP, our results support the use of a single colonic biopsy as an original source of biomarkers in PD beyond the sole assessment of Lewy pathology.

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Immunoblots of colonic biopsies and brain samples lysates diluted at 1 : 10 with GFAPmono, C-Term, and N-term antibodies.

Figure S2. Representative immunoblots of colonic biopsies lysates showing the heterogeneous phosphorylation of GFAP at serine 13 between PD patients.

Figure S3. GFAP phosphorylation at serine 13 in colonic biopsies lysed in NETF buffer.

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