

## Live monitoring of brain damage in the rat model of amyotrophic lateral sclerosis

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**Abstract.** Amyotrophic lateral sclerosis (ALS) is a devastating neurological disorder affecting upper and lower motoneurons. The transgenic ALS rat model (hSOD-1<sup>G93A</sup>) was used for magnetic resonance imaging (MRI) study using a low field wide bore magnet. T2-weighted hyperintensities were observed in the brainstem, rubrospinal tract and vagus motor nuclei with prominent lateral ventricle and cerebral aqueduct enlargements. These changes could be observed already in presymptomatic animals. T2\*-weighted MRI with magnetically labeled antibodies (against CD4) revealed lymphocyte infiltration in the brainstem-midbrain region corresponding to the areas of dilated lateral ventricles. Confocal imaging revealed reactive astroglia in these areas. Thus, with the use of wide bore MRI new sites of neurodegeneration and inflammation were revealed in the hSOD-1<sup>G93A</sup> rat model.

**Key words:** Amyotrophic lateral sclerosis — Magnetic resonance imaging — Ultra small particles of iron oxide — hSOD-1<sup>G93A</sup> rat model — CD4 cells — Astrogliosis

### Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequently encountered primary form of progressive motoneuron disease. It is a devastating neurological disorder affecting upper and lower motoneurons. It is characterized by progressive muscle weakness and atrophy. It is almost invariably fatal, usually within 3 to 6 years after the beginning of the symptoms.

ALS tissue studies reveal microglial activation as well as other markers of inflammatory processes in both human and transgenic mouse models (Alexianu et al. 2001; Appel and Simpson 2001; McGeer and McGeer 2002; Henkel et al. 2004). Moreover, neuroimmunologic evidence also exists for the systemic immunologic activation in sporadic ALS patients (Zhang et al. 2005). Flow cytometry of immune cell markers has thus revealed activated monocyte/macrophages, the degree of activation being directly correlated to the rate of

disease progression. In addition, disease associated changes were also observed for parameters of T-cell activation and immune globulin levels. In a recent study of the role of individual cell types in the murine superoxide dismutase 1 (SOD-1)-based ALS the mutant gene SOD-1<sup>G37R</sup> was selectively “turned down” in motor neurons or in microglia (Boillee et al. 2006). In addition to a substantial delay of onset and progression of the disease in motor neurons with suppressed mutant SOD-1, selective diminution of the mutant enzyme in microglia greatly delayed the progression of the later disease stage. Thus, the initiation role of microglia in the final stage leading to the demise of motor neurons in ALS was underlined. However, it could not be excluded that the slowing of the later disease phase may have also been caused in part by gene inactivation in peripheral macrophages or their progenitors and/or from the migration of those cells into the central nervous system after initial motor neuron damage.

Magnetic resonance imaging (MRI) as a non-invasive technique is becoming a preferred neuroimaging technique for the diagnosis of ALS since this is the only technique that can access the degenerative processes inside the upper motor cortex. ALS as “multi-focal system syndrome” is character-

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ized by the degeneration of motor neurons followed by the loss of myelinated fibers which are clearly manifested as hyperintense areas in T2-weighted MRI (Ellis et al. 1999; Basak et al. 2002). However, a number of other neurological diseases can produce similar MRI findings. Numerous standard and advanced MRI techniques including diffusion imaging and magnetic resonance spectroscopy have been used to improve diagnostic capabilities and enhance specificity of the MRI examination (Hecht et al. 2002, Suhy et al. 2002; Sach et al. 2004), but it became clear that no further progress can be achieved without the reliable animal model where MRI findings can be compared to the pathological findings and putative therapeutic approaches can be tested in longitudinal studies. Transgenic mice overexpressing mutated human SOD-1 (hSOD-1) provided the first and still widely used animal model of familial ALS (Gurney 1997). In this model the foci of degeneration in brainstem and lumbar spinal cord have been shown to be similar to the findings in human pathology (Lowry et al. 2001). An MRI study of the transgenic mouse model (Zang et al. 2004) also revealed degenerative changes in the brain and cerebellum resembling findings in human MRI studies, but their findings were not very informative in terms of revealing underlying mechanisms.

Recently a transgenic rat model of ALS that expresses multiple copies of hSOD-1 carrying the G93A missense mutation (hSOD-1<sup>G93A</sup>) has been developed (Howland et al. 2002) and it has been observed that this model of ALS exhibits electrophysiological and morphological features also manifested in human ALS (Philippeau et al. 2005). Due to the larger size as compared to the mouse model the hSOD-1<sup>G93A</sup> transgenic rat allows for better phenotypic characterization, in particular more reliable use of physiological and biochemical techniques. Larger size of animals is also advantageous for MRI studies but no MRI study has been performed so far on this rat model presumably because of the common belief that specialized MRI machines are required. In line with the advantages of the rat model we have undertaken an MRI study of its brain in symptomatic and presymptomatic animals as compared to wild-type (WT) using clinical MRI and matching these findings with confocal microscopy of immunostained brain slices. In order to enhance the specificity of MRI evaluation and to demonstrate that an infiltration of inflammatory cells occurs in the neurodegenerative foci *in vivo* we also used CD4 antibodies commercially available as magnetically labeled with ultra small particles of iron oxide (USPIO). These paramagnetic nanoparticles are detectable through their property of generating strong susceptibility effects. Recently, several groups have reported success with immune cell-specific imaging, using superparamagnetic contrast materials that are either internalized by cells after *ex vivo* incubation, or bound to the cells by specific antibodies (Anderson et al. 2004; Bulte and Kraitichman 2004). Apply-

ing this approach for the first time in the ALS rat model we aimed to open doors for a better understanding of inflammatory infiltration through live tissue monitoring in this neurodegenerative disease.

## Materials and Methods

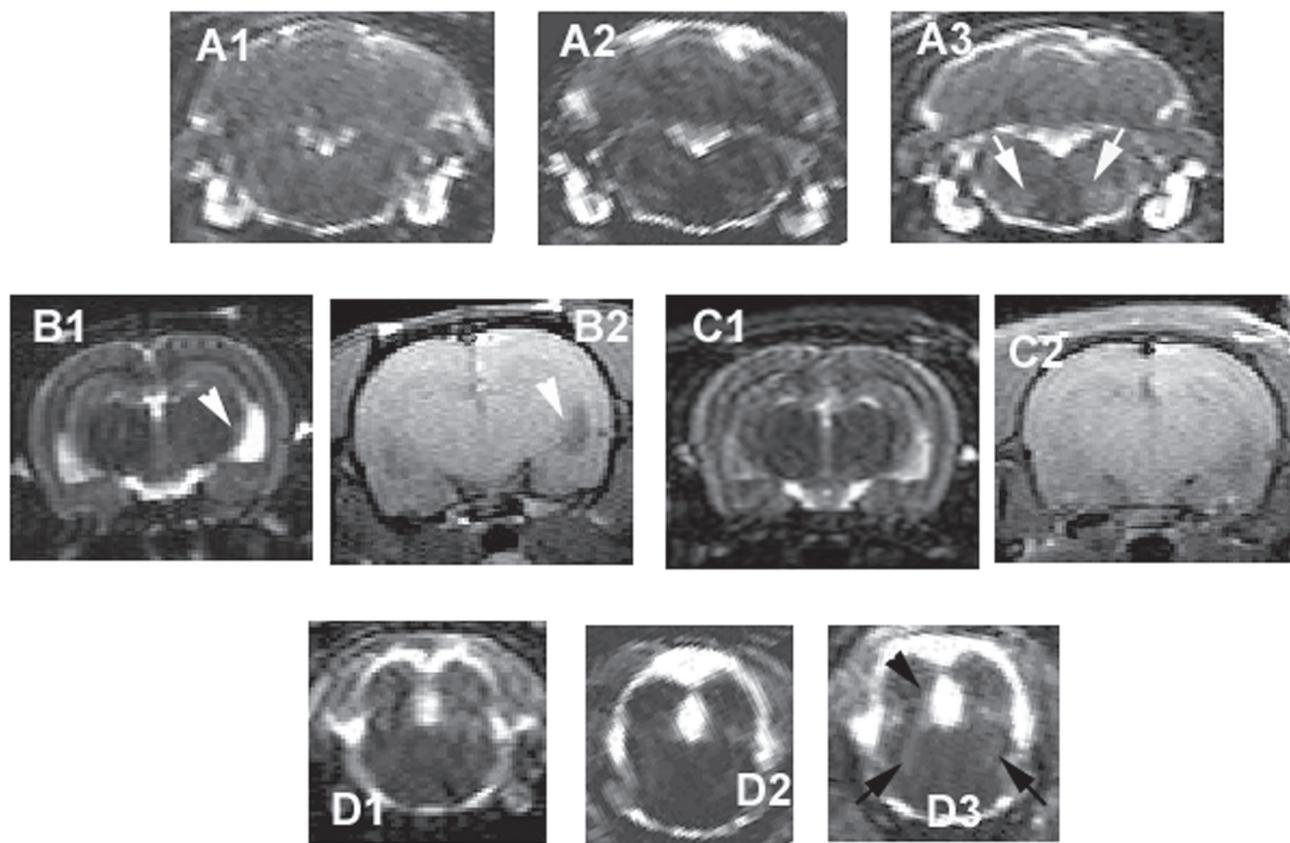
### Animals

Experiments were performed on Sprague-Dawley rats: genetically modified, expressing multiple copies of mutated (G93A) human SOD-1 gene (hSOD-1<sup>G93A</sup>; Taconic Farms Inc. NY) and WT (standard Sprague-Dawley rats; kind gift from Dr. J. Borota). The disease was expressed 7 to 8 months upon birth and  $20 \pm 1$  day ( $n = 7$ ) was the period from the onset to the disease end-stage (mean age at end-stage was  $254 \pm 11$  days,  $n = 8$ ). The disease process was followed by visually checking the movement of the animal on a flat surface and by testing the regaining of stature after turning to the side and if the latter was not possible the end-stage of the disease was indicated and the animal was sacrificed for histology.

### MRI

Animals were imaged in different stages of the disease – presymptomatic, symptomatic and end-stage. Rats were anaesthetized by intraperitoneal (i. p.) injection of 0.045 g/ml Nembutal (45 mg per kg body weight) and placed in the prone position for brain and brainstem imaging. Animals were treated in strict accordance with the European Communities Council Directive (86/609/EEC) and with approval of the Ethical Committee of the Faculty of Biology University of Belgrade. Accordingly all efforts were made to minimize animal suffering and to reduce the number of animals used. Standard T1-weighted (T1W) and T2W images of rats were obtained using the clinical 1.5 T Avanto MRI imager (Siemens) with a 3 cm mini surface radio frequency (RF) coil placed over the skull. Images were obtained using the Turbo Spin Echo T2W sequence with repetition time TR = 5800 ms; echo time TE = 200 ms, and the slice thickness of 1.5 mm (voxel dimensions  $1.5 \times 0.2 \times 0.3$  mm) and T1W imaging with TR = 400 ms, TE = 23 ms. The total scanning time including preparation of animal for imaging was about 45 min. Such relatively short scanning time allowed for work without supplemental body heating. Following MRI scans, rats were warmed and monitored until they recovered fully.

In order to check for the infiltration of inflammatory cells in the brain tissue antibodies against CD4 cells (mostly T cells and some macrophages and monocytes (Jefferies et al. 1985)), magnetically labeled with USPIO (MACS<sup>®</sup>, Miltenyi Biotec) were i.p. injected into rats (200  $\mu$ l of original solu-

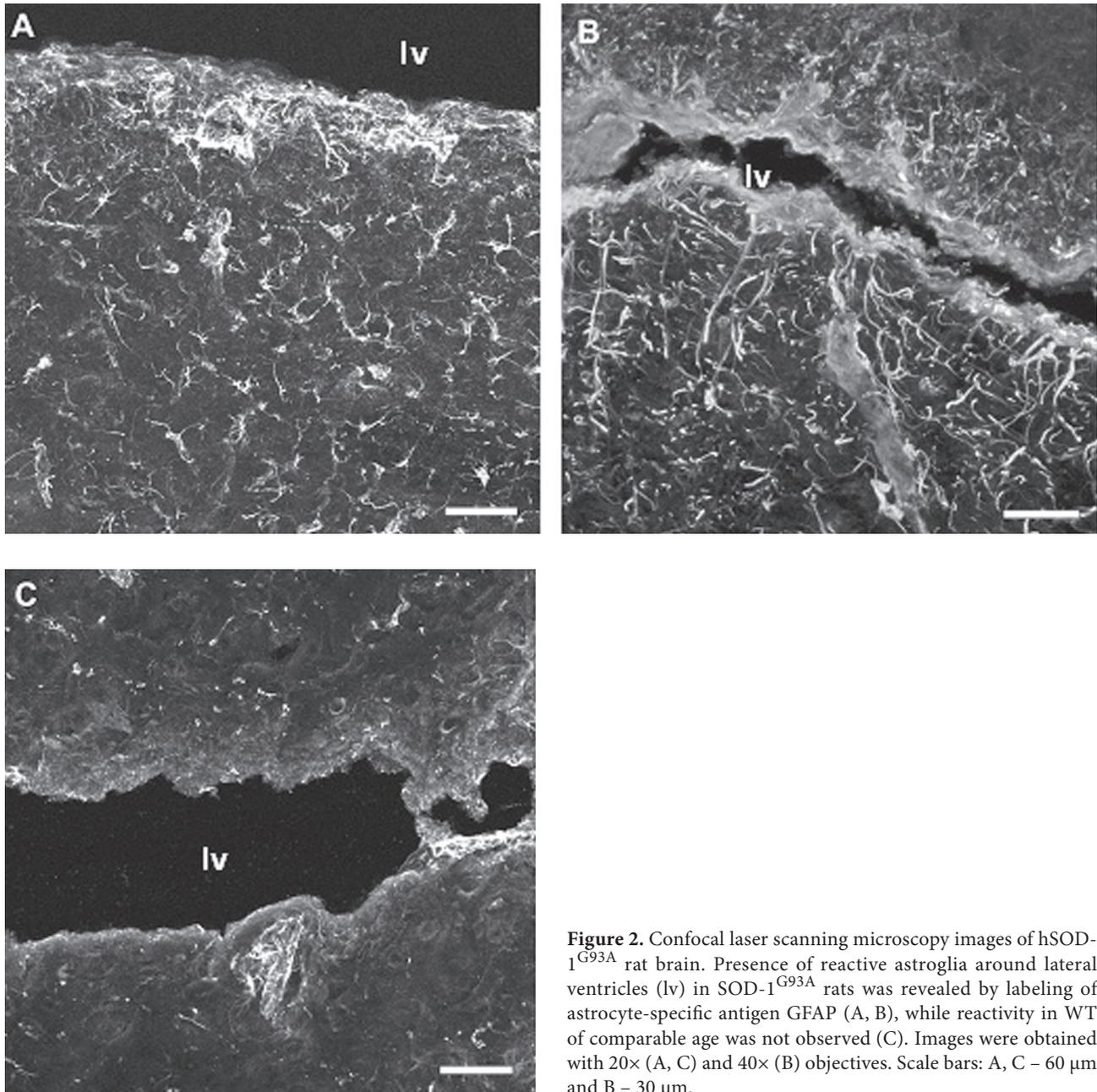


**Figure 1.** Coronal MRI of the brainstem of WT and presymptomatic and symptomatic hSOD-1<sup>G93A</sup> rats at different levels. Hyperintensity can be observed in two cranial nerve nuclei: nucleus ambiguus and motor trigeminal nucleus, as well as in the paragigantocellular nucleus in the presymptomatic (243-days-old; A2) and in the disease end-stage (same animal 272-days-old; A3 – arrowheads), but not in WT of comparable age (A1). T2W and T1W MRI showed significant enlargement of the lateral ventricles at the end-stage in hSOD-1<sup>G93A</sup> rats (B1 and B2, respectively, another animal, note arrowheads) as compared to WT (C1 and C2). Also note at the level of pons hyperintensity in the area of the rubrospinal tract – presymptomatic (D2) and symptomatic (D3), and an enlarged aqueduct (same animal as in A) as compared to WT (D1).

tion in 1 ml of physiological saline). Animals were injected 24 h prior to imaging, estimated as optimal incubation time for such studies (Floris et al 2004; Pirko et al. 2004). The anaesthetized animals were then scanned using the clinical 3.0 T MRI magnet (Intera, Phillips) with two small surface RF coils placed laterally at each side of the animal head (slice thickness – 2 mm, number of averages – 6, phase encoding number – 256, field of view – 100 mm, acquisition matrix – 256). Images were taken using the Gradient Echo T2\*-weighted sequences (TR = 50 ms) and two different TE (20 ms and 30 ms) were used to confirm that the observed artifacts (signal voids) originate from uptaken USPIO-labeled antibodies. The specific small size and optimal filling factor of the excitation RF coils (Pirko et al. 2004) used in both MRI setups as well as the larger rat model as compared to the mouse allowed for obtaining good resolution even with a wide bore magnet.

#### Immunofluorescence

Brains were dissected from animals deeply anaesthetized with i. p. injection of Nembutal and fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer pH 7.4 overnight at 4°C. Cryoprotection was achieved by immersion in 30% sucrose in 0.1 mol/l phosphate buffer. The brains were cut on a cryostat in 50 µm-thick coronal slices that were mounted on gelatinised microscope slides and dried overnight at room temperature. Slices were treated with 10% goat serum in PBS for 30 min and then incubated overnight at 4°C with primary antibodies in 2% goat serum in 0.01 mol/l PBS. Cell antigens were labelled for astrocytes with mouse monoclonal anti-gliofibrillary acidic protein (GFAP; 1 : 100, Molecular Probes Eugene). After wash in 0.01 mol/l PBS slices were incubated 2 h with secondary antibodies diluted in PBS. Visualization of primary labelling was done with Alexa Fluor 555-conjugated goat anti-mouse



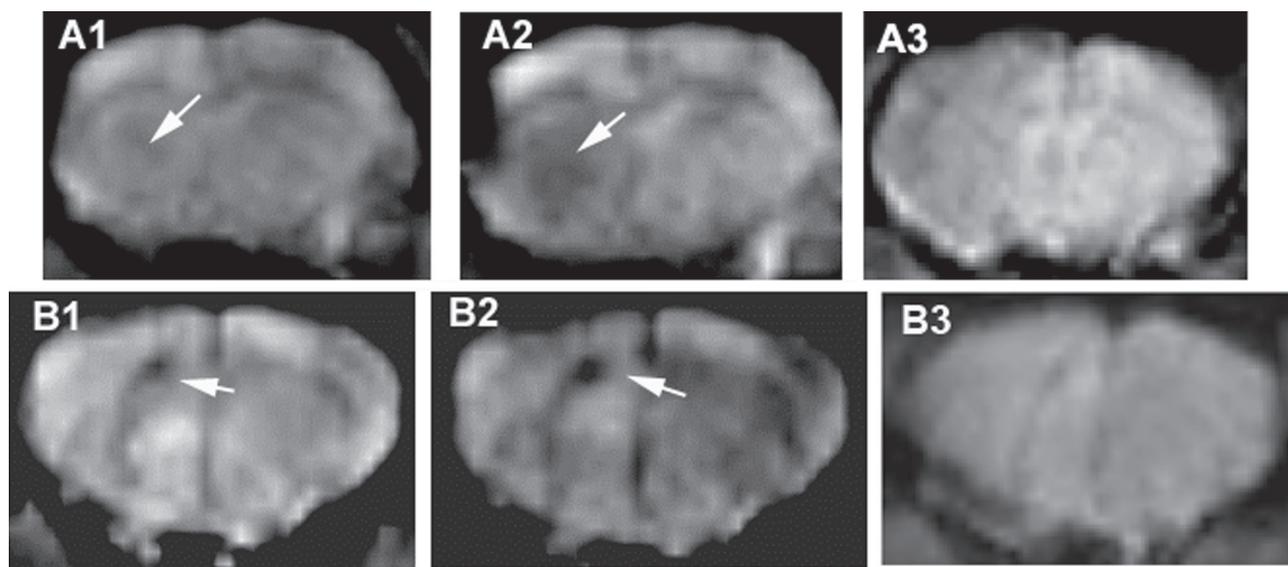
**Figure 2.** Confocal laser scanning microscopy images of hSOD-1<sup>G93A</sup> rat brain. Presence of reactive astroglia around lateral ventricles (lv) in SOD-1<sup>G93A</sup> rats was revealed by labeling of astrocyte-specific antigen GFAP (A, B), while reactivity in WT of comparable age was not observed (C). Images were obtained with 20× (A, C) and 40× (B) objectives. Scale bars: A, C – 60 μm and B – 30 μm.

antibodies (1 : 200, polyclonal, Molecular Probes). The stained sections were embedded with glycerol in PBS, glass covered and finally examined using LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

## Results and Discussion

In eight transgenic animals and two WT rats of comparable age MRI coronal images were obtained from the frontal

cortex towards the brainstem medulla. As compared to age matched WT animals, hyperintensive areas in the tissue indicative of foci of neurodegeneration, were found in the brainstem of the G93A rats (Fig. 1). This was most apparent on T2-weighted images in the areas corresponding to the trigeminal and vagus motor nuclei, trigeminal spinal tract, parvicellular and paragigantocellular reticular nucleus, and nucleus ambiguus (see example in Fig. 1A3). These findings were congruent with the MRI study on the murine model (Zang et al. 2004). In the present study, however, hyperin-



**Figure 3.** MRI of the brain of SOD-1<sup>G93A</sup> rat at two disease stages (A – 222 and B – 215 days) injected with USPIO-tagged antibodies against CD4. For the midbrain region T2W images with TE = 20 ms revealed dark areas in the vicinity of the lateral ventricle (A1 – arrow) that became more apparent with TE = 30 ms (A2 – arrow). The latter protocol does not reveal a similar signal void in WT of comparable age (A3). Rostrally to the midbrain region in the vicinity of the lateral ventricle an apparent USPIO signal artefact was also observed with TE = 20 ms (B1 – arrow) and became more apparent with TE = 30 ms (B2 – arrow) with no similar effect of the latter protocol in the WT (B3).

tensive foci not observed in WT animals (Fig. 1A1) were already observable in presymptomatic animals (Fig. 1A2 – 243-days-old animal). Specifically for the rat model MRI also revealed neurodegeneration in the rubrospinal tract and vagus motor nuclei (presymptomatic vs. symptomatic – Fig. 1D2 vs. D3 – 29 days apart). However, the most apparent features of brain tissue atrophy observed in this model were the widespread enlargements of the lateral ventricles, clearly noted on T2W (Fig. 1B1, hyperintense regions) as well as on T1W images (Fig. 1B2, hypointense regions), not observed in WT (Fig. 1C), and a dilated cerebral aqueduct (Fig. 1D3). The latter phenomenon has previously been observed in the murine model (Zang et al. 2004) but was revealed here also in presymptomatic cases (see Fig. 1D2) as compared to the lack of such appearances in age related WT cases (Fig. 1D1).

Regardless of the pulse sequences employed it is difficult to detect inflammatory and neurodegenerative foci near ventricles since those tissues would appear hyperintense in T2W and hypointense in T1W images and may be mixed with the signal from the cerebro-spinal fluid. In order to check for the changes in the brain tissue close to the dilated lateral ventricles brain slices from end-stage animal models were immuno-labelled for the astroglial marker – GFAP. By means of laser scanning confocal microscopy this immunocytochemical approach revealed reactive astrogliosis in the vicinity of lateral ventricles (Fig. 2). Namely, in ALS

preparations an intense GFAP signal was observed revealing astrocytes with enlarged cell bodies and extended thick processes. Such reactive astrocytes became much denser in close proximity of the lateral ventricles.

It would greatly enhance the progress of ALS research if inflammatory processes associated with ALS could be more specifically imaged by MRI. In fact, among different immune system alterations in ALS patients it was observed that the percentage of CD4 expressing lymphocytes was increased (Zhang et al. 2005). Thus, in order to follow the inflammatory cells in ALS brain tissue we chose to use commercially available antibodies against CD4 receptors tagged with USPIO nanoparticles. These were i.p. injected to G93A animals in the symptomatic, late phase of the disease and compared to age matched WT animals treated with the same dose of CD4 USPIOs. A similar procedure has been used in studies on experimental allergic encephalomyelitis, the animal correlate of multiple sclerosis (Floris et al. 2004; Pirko et al. 2004). However the latter experimental procedures of identifying the location of uptaken USPIO particles were rather elaborate and questionable in terms of specificity. Here we used the MRI protocol that employs the fact that accumulated superparamagnetic nanoparticles, USPIOs induce susceptibility artefacts that are manifested as a signal void (dark spots) in MR images. The area of the signal void is larger than the actual size of the tissue that accumulates USPIOs and this effect depends on the particular MRI pulse sequence. To

make sure that the signal void in MR images arises from accumulated nanoparticles – USPIOs, we performed two consecutive gradient echo T2\* images with different values of echo times (20 or 30 ms). Prolonging the echo time in the gradient echo sequence augments the signal artefact generated by the magnetic particles, thus comparison of images obtained using different echo times becomes a reasonably good sign of USPIO presence in the tissue. We also used a 3.0 T MRI instrument since the susceptibility-induced artefacts increase with the magnetic field strength. In addition to the T2\* imaging, the obtained signal artefact could also be double-checked with T1W imaging that would usually give a hyperintensity in the congruent region (not shown). The USPIO-generated T2\* signal void was studied mainly in the midbrain region in the vicinity of lateral ventricles (Fig. 3). This USPIO-generated effect was also recorded for the tissue around the lateral ventricle more rostrally to the midbrain region (Fig. 3B). These images point to a general phenomenon in the ALS model – infiltration of inflammatory cells (lymphocytes) into the brain tissue in the vicinity of the lateral ventricles. A direct live monitoring of immune cell markers is thus demonstrated allowing for further studies with additional magnetically labelled antibodies that should address the topology of specific cells. It is likely that the lymphocyte infiltration is a consequence of a compromised blood brain barrier as recently indicated for the mouse model (Garbuzova-Davis et al. 2007). The latter can be assessed by using contrasting agents such as Gd-DTPA (Floris et al. 2004) in conjunction with MRI protocols used here.

In conclusion, using clinical MRI we managed to assess neurodegenerative processes in the hSOD-1<sup>G93A</sup> transgenic ALS rat model comparable to the images obtained for the mouse model at 4.7 T (Zang et al. 2004). Additional foci of neurodegeneration were also observed with the lateral ventricle dilation as the most apparent feature of brain tissue atrophy. Degenerative processes in these areas were also confirmed by confocal images of reactive astroglia. By means of a wide bore 3.0 T MRI and anti-CD4 antibodies tagged with magnetic nanoparticles it was also demonstrated that in the vicinity of the dilated ventricles an accumulation of inflammatory cells occurs. Thus, in the hSOD-1<sup>G93A</sup> transgenic rat model of ALS neuroinflammatory markers were revealed also justifying future studies on specific cell populations with different magnetically labeled antibodies and on the role of blood brain barrier with MRI contrasting agents.

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