Persistent activation of microglia is associated with neuronal dysfunction of callosal projecting pathways and multiple sclerosis-like lesions in relapsing—remitting experimental autoimmune encephalomyelitis

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Cortical pathology, callosal atrophy and axonal loss are substrates of progression in multiple sclerosis (MS). Here we describe cortical, periventricular subcortical lesions and callosal demyelination in relapsing-remitting experimental autoimmune encephalomyelitis in SJL mice that are similar to lesions found in MS. Unlike the T-cell infiltrates that peak during acute disease, we found that microglia activation persists through the chronic disease phase. Microglia activation correlated with abnormal phosphorylation of neurofilaments in the cortex and stripping of synaptic proteins in cortical callosal projecting neurons. There was significant impairment of retrograde labeling of NeuN-positive callosal projecting neurons and reduction in the labelling of their transcallosal axons. These data demonstrate a novel paradigm of cortical and callosal neuropathology in a mouse model of MS, perpetuated by innate immunity. These features closely mimic the periventricular and cortical pathology described in MS patients and establish a model that could be useful to study mechanisms of progression in MS.

Keywords: EAE; callosal projecting neurons; microglia; MS; neurodegeneration; live imaging

Abbreviations: MS = multiple sclerosis; NAWM = normal-appearing white matter

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Introduction

A key feature of progression in multiple sclerosis (MS) is the development of widespread neuronal dysfunction in cortical and subcortical areas (Imitola *et al.*, 2006), together with lesions characterized by demyelination, inflammation and axonal loss (Lassmann, 1998). Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model for MS, but murine EAE models are thought to have predominant spinal cord pathology and to rarely affect the brain (Waksman and Adams, 1962). Cortical lesions mimicking those in MS have previously been demonstrated in the marmoset EAE model (Villoslada *et al.*, 2000; Pomeroy *et al.*, 2005; Merkler, Boscke *et al.*, 2006), but the high cost and demanding requirements for animal husbandry as well as the absence of knockout or transgenic animals make the availability of a rodent model of crucial interest. Cortical lesions can be induced in Lewis rats by cortical injection of proinflammatory cytokines (Merkler, Ernsting *et al.*, 2006), but these lesions are reversible and the model is not suitable for investigating questions about the chronic phase of MS (Steinman, 2001).

MS starts as a focal inflammatory disease of the CNS associated with demyelinating plaques (Ge et al., 2005),

but progression of the disease is thought to be related to chronic activation of parenchymal and perivascular microglia (Kutzelnigg et al., 2005). This is supported by data from the EAE model where inactivation of microglia inhibits EAE (Heppner et al., 2005). Microglia-mediated neurotoxicity has been well described in vitro, where activated microglia trigger the production and release of neurotoxic molecules and pro-inflammatory cytokines that lead to severe neuronal dysfunction through axonal loss (Cardona et al., 2006; Skaper et al., 2006). In vivo neuronal toxicity with synaptic dysfunction during EAE has been correlated with the appearance of inflammatory infiltrates and activated microglia (Zhu et al., 2003; Marques et al., 2006). The mechanism of synaptic dysfunction is thought to be related to microglia separating pre- and post-synaptic nerve terminals (Blinzinger and Kreutzberg, 1968). In MS brain, activated microglia were found closely apposed and ensheathing apical dendrites, neurites and neuronal perikarya, and in association with transected axons and neuritic ovoids (Peterson et al., 2001).

Cortical pathology and callosal atrophy are associated with progression of MS (Filippi *et al.*, 2003; Kutzelnigg *et al.*, 2005; Martola *et al.*, 2006). Callosal projection neurons located primarily in layers II/III, V and VII in adult neocortex connect homotopic areas from the two cerebral hemispheres through the corpus callosum, which has a central role in inter-hemispheric communication (Mitchell and Macklis, 2005; Martola *et al.*, 2006). Callosal projection neurons are thought to be important for motor coordination and as well as cognitive processes (Rouiller *et al.*, 1994; Hampel *et al.*, 2002), both of which are impaired in chronic progressive MS.

We used a relapsing-remitting EAE model to investigate neuronal dysfunction of cortical callosal projecting pathways in relation to activated microglia. The model shows features that resemble some of the periventricular and cortical pathology described in MS patients. We also demonstrate a novel paradigm of cortical and callosal neuropathology affecting callosal projection neurons by activated microglia. These data establish a murine model that could be useful for investigating the pathogenesis of progression and potential therapeutic approaches for chronic MS.

Materials and methods

Animals

Sixty female SJL/J mice were purchased from Jackson Laboratories Inc. (Bar Harbor, ME) and used at 6–8 weeks of age. Mice were housed 4/cage and maintained on a 12-h light/dark cycle with food and water ad libitum. Upon arrival, mice were randomly assigned to experimental groups, followed by an acclimation period of at least 3 days prior to any experiment.

EAE induction

SJL/J mice were immunized subcutaneously in two sites (left and right flank) with $150 \,\mu g$ of PLP139-151 (New England Peptide LLC, Gardner, MA) emulsified in complete Freund's

adjuvant (CFA, Sigma Aldrich, Saint Louis, MO) containing 200 µg *Mycobacterium Tuberculosis* (Difco Laboratories, Detroit, MI). Mice received 200 ng pertussis toxin (PT, List Biological Laboratories Inc., Campbell, CA) in 0.2 ml PBS (Lonza, Walkersville, MD) intraperitoneally (ip) at the time of immunization and 48 h later. Control mice were immunized with CFA followed by PT. Mice were scored daily for neurological signs as follows: 0, no disease; 1, loss of tail tone; 1.5, poor righting ability; 2, hind limb weakness; 3, hind limb paralysis; 4, scale 3 plus forelimb weakness; 5, moribund.

Criteria for selection of time points

The mice were sacrificed at different time points and divided into four groups depending on the course of disease and clinical score. Defining the time points was not based on the number of days but rather on the course of the disease in individual animals. Acute phase (around 17 days post immunization, dpi) when animals reach a minimum clinical score of 1.5; first remission (around 32 dpi) with a maximum clinical score of 1 after the acute phase; late relapse (between 90–100 dpi) where animals have to reach a minimum clinical score of 1.5; late remission (between 90 and 100 dpi) where the clinical score has to drop back down to a maximum of 1 after two relapsing phases.

Tissue processing

At the appropriate time points the mice were deeply an aesthetized in a $\rm CO_2$ chamber and transcardially perfused with cold PBS followed by 4% cold paraformal dehyde solution (PFA, Electron Micoscopy Sciences, Hatfield, PA) in PBS. Brains were removed and post-fixed in PFA for 48 h.

Histology

The brains were embedded in paraffin and sliced into 2-µm-thick coronal sections and stained using haematoxylin and eosin (H&E) for infiltrating cells, Luxol Fast Blue (LFB) for demyelination or Bielschowsky stain for axonal damage.

Histological evaluation

Number of inflammatory foci, identified as perivascular clusters containing at least 20 mononuclear cells were quantified. The mean number of foci was calculated by averaging the counts from eight sections (four consecutive sections at bregma 0.0 and four consecutive sections at bregma -0.02, Franklin and Paxinos, 1997) according to their location; leukocortical (involving both white matter and adjacent cortical grey matter), intracortical (entirely within cortex) or subpial. Areas with loss of LFB parenchymal staining indicative of demyelination or loss of Bielschowsky staining indicative of axonal loss were compared with similar areas in age-matched controls. The tissue damage in the white matter lesions was graded as normal (grade 0), disarrangement of the nerve fibres (grade 1), the formation of marked vacuoles (grade 2) and the disappearance of myelinated fibres (grade 3) as previously described (Wakita *et al.*, 1994).

FACS analysis of CNS isolated cells

Mice were deeply anaesthetized in a CO_2 chamber and transcardially perfused with 30 ml PBS. The corpus callosum and cortical tissue was dissected from a 2 mm block from

2818 Brain (2007), **130**, 2816–2829

the forebrain, minced in a 70 μ m strainer, and collected in HANKS media (Invitrogen, Eugene, OR) containing 5% fetal bovine serum (Invitrogen) and 0.05% NaN₃ (Sigma Aldrich). The cells were enzymatically dissociated in HBSS (Lonza) containing 10 mM HEPES (Lonza) and 2 mM EDTA (Sigma Aldrich) for 1 h at 4°C. Cells were then washed and resuspended in 37% Percoll (Amersham Biosciences Corporation, Piscataway, NJ) and centrifuged for 10 min at 500 g to separate cells from myelin. Cells were labelled directly with fluorescent antibodies (all from BD Biosciences, San Jose, CA) for 30 min for anti-CD4-FITC (1:100), anti-CD8-PE (1:100), anti-CD45-PerCP-Cy5 (1:100) and anti-CD11b-APC (1:100), washed three times and fixed in 1% PFA for 10 min, and analysed by a four-colour flow cytometer on a FACS-Calibur (Becton Dickinson, Mountain View, CA). The data was analysed using FlowJo 3.7.1 software program.

Immunohistochemistry

Brains were placed in 30% sucrose for at least 24 h for cryoprotection. Coronal blocks of brain tissue from bregma -2to +2 mm were frozen in cryo-protective O.C.T.-solution (Sakura Finetek, Torrance, CA) at -80° C. The tissue was cut into floating sections of 40 µm thickness on a freezing microtome. Floating sections were blocked with 8% horse serum for 1 h and incubated overnight with mouse anti-NeuN antibody (1:100, Chemicon, Temecula, CA), rat anti-CD4 (1:100, BD Biosciences), rat anti-CD8 (1:100, BD Biosciences) and rabbit anti-activated Caspase-3 antibody (1:250, BD Biosciences) or rat anti-CD11b (1:20, BD Biosciences) and one of the following antibodies: rabbit anti-CTFG antibody (1:500, Abcam Inc., Cambridge, MA), rat anti-Ctip2 antibody (1:500, Abcam Inc.), goat anti-Cux-2 antibody (1:10, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-MeCP2 antibody (1:500, Affinity Bioreagents, Golden, CO), mouse anti-SMI-32 (non-phosphorylated neurofilaments) antibody (1:250 Sternberger Monoclonals Inc., Lutherville, MD) or mouse anti-MBP antibody (1:2000, Sternberger Monoclonals Inc.). Sagittal brain sections were stained for mouse anti-β-tubulin III antibody (1:100 Stem cells Technologies). Sections were rinsed and incubated for 1 h with the appropriate Alexa Flour 488 and 594 secondary antibodies (1:500, Molecular Probes, Eugene, OR) and mounted on microscope slides and cover-slipped. Negative control sections for each animal received identical preparations for immunostaining, except that primary antibodies were omitted.

TUNEL staining was performed according to manufacturer's instructions using an *In Situ* Cell Death detection kit from Roche Applied Systems (Roche Applied Sciences, Mannheim, Germany). Briefly, deparaffinized sections were incubated for 15 min at 37° C in a humidified chamber in Proteinase K solution (20μ g/ml in 10 mM Tris pH 8.0, Roche Applied Sciences). After a washing step, sections were incubated with the enzyme solution diluted 1:10 in labelling solution. For double labelling with NeuN and TUNEL, the sections were immunostained first and fixed with 4% PFA/PBS for 15 min at RT. After a washing step, sections were stained for TUNEL as described earlier omitting the permeabilization with proteinase K.

Confocal analysis

Regions of interest were analysed with a confocal microscope (LSM 510 Laser Scanning Microscope and LSM 3D analysis software, Linux, Ogdensburg, NY) with a $20 \times$ air-immersion objective lens for expression of CD11b/SMI-32 and quantification

of CD11b. To determine the morphology of microglia cells, expression of synaptophysin and cell–cell interaction of microglia and neurons a 63× water-immersion objective lens was used. The LFB and the MBP covariance were measured as the pixel intensity over a distance of 300 μ m in the corpus callosum. The density of the callosal projecting axons was determined by the mean pixel intensity on a scale from 0 to 255 of the β -tubulin III (10 squares of 0.0214 mmxmm) in the genu of the corpus callosum with a 63× water-immersion objective lens. TUNEL and activated Caspase-3 positive cells were stained for NeuN and the number of single and double positive cells was quantified per brain slide.

Confocal live imaging of microglia

Tissue of interest from cortex and corpus callosum from chronic EAE mice 210 dpi and age-matched controls was dissected out and sliced at a thickness of 200 µm using a McIlwain tissue slicer (Brinkman). Fluorescent staining of microglia in living brain tissue slices was performed with Alexa 488 IB₄ (Molecular Probes) in a six-well plate with 30-mm culture plate inserts (Millipore Coporation, Billerica, MA) by adding a stock solution to the culture medium at a final concentration of 5 µg/ml for 60 min. Before recording, the slices were washed, transferred to a glass bottom 30 mm petri dish, covered with 200-500 µl of growthfactor reduced matrigel (BD biosciences) and gelled at 37°C for 15 min to minimize tissue motion during recording. The movies were recorded within the first hour after the IB₄ staining was completed to avoid unspecific microglia activation, with a confocal microscope with a $63 \times$ water-immersion objective lens (LSM 510 Laser Scanning Microscope, Linux, Ogdensburg, NY) as z-stacks taken every 5 min for a total of 1 h (Dailey and Waite, 1999; Stence et al., 2001).

Neuronal tracing studies

Mice were deeply anaesthetized with ketamine/xylazine, then secured in a stereotaxic mouse surgical frame (Stoelting). Callosal projection neurons with projections to the contralateral neocortex were labelled with hydroxystilbamidine (2% in ddH₂O, Molecular Probes), an equivalent to FluoroGold according to Molecular Probes and previous research (Cheunsuang *et al.*, 2006). The mice received 20 injections in an area of 3×3 mm, 50 nl per site (craniotomy extending A/P from +1.0 mm to -2.0 mm from bregma; M/L 0.5 to 3.5 mm lateral to bregma; Franklin and Paxinos, 1997). Two days after the injection of hydroxystilbamidine, mice were deeply anaesthetized in a CO₂ chamber and transcardially perfused with cold PBS followed by 4% cold PFA in PBS. Brains were removed and postfixed in PFA for 48 h.

Counting of callosal projecting neurons

Forty micrometre sections were cut on a freezing microtome, and stained for NeuN as described previously. Twenty consecutive sections extending from bregma 0.0 to -0.8 mm (Franklin and Paxinos, 1997) were used for neuron counting. Labelled neurons were quantified and categorized within cortical layers II/III, V and VII. The pixel intensity of the axons in the corpus callosum was measured over a distance of 200 µm together with the intensity of the staining of the radiating axons extending out from the genu of the corpus callosum. Sections were examined under epifluorescence with a Zeiss Axioplan with high-numerical-aperture objective lenses. Quantification of neurons was

performed with a 40× oil-immersion objective lens, and the pixel intensity of the corpus callosum and the radiating axons extending out from the genu of the corpus callosum was measured with a $25\times$ oil-immersion lens.

Statistical analysis

All data is presented as mean \pm SEM. Statistical analysis was performed using the unpaired, two-sided *t*-test comparison between EAE and control or by using one-way analysis of variance (ANOVA) with Turkey's Multiple Comparison Test. Significant differences were assumed at the 5% level and represented as *P*-values (*P* < 0.05).

Results

Forebrain pathology in SJL mice during EAE is reminiscent of MS pathology

SJL/J mice immunized with PLP 139-151 develop relapsingremitting EAE, with onset of clinical signs occurring on day 11-12 post immunization and peaking around day 17. The mice then enter a remission phase around day 30-35 followed by several relapses and remissions (Fig. 1a). The mice were then divided into four groups depending on the course of disease and clinical score; acute (n = 20) average clinical score 1.575 ± 0.05 ; first remission (n = 13)average clinical score 0.269 ± 0.11 ; late relapse (n = 21) average clinical score 2.143 ± 0.11 and late remission (n=14) average clinical score 0.821 ± 0.09 (data not shown). On average the duration of a relapse was 33 ± 9 days and the duration of remission was 8 ± 4 days (data not shown). In contrast to other models of EAE, we found a dramatic number of lesions throughout the forebrain in the immunized SJL/J mice (Fig. 1), with the typical perivenular accumulation of mononuclear cells (Lassmann et al., 1981). We found inflammatory lesions (defined as a cluster of at least 20 mononuclear cells) correlating with cortical and white matter (WM) pathology previously described for MS and EAE (Peterson et al., 2001; Pomeroy et al., 2005; Merkler, Ernsting et al., 2006; Wegner et al., 2006). Leukocortical lesions involving both white matter and adjacent cortical grey matter, were of variable size and shape (diameter: 54-682 µm), and often found centred on a vessel in cortical layer VII (Fig. 1d). Intracortical inflammatory lesions were small and round or oval in shape (diameter: $65-137 \,\mu\text{m}$) and were always centered on a vessel (Fig. 1e). We also found a number of subpial lesions located in the superficial cortical layers running parallel to the cortical surface (Fig. 1f). These lesions could cover a very large area (up to 1240 µm in diameter). All three types of neocortical inflammatory lesions and WM inflammatory lesions in the corpus callosum (Fig. 1g) were detected in all mice examined, while few inflammatory foci were observed in CFA immunized control mice (Fig. 1h-k). The total number of inflammatory foci was increased 7-fold in acute animals compared to controls and remained high throughout the disease (all had a P < 0.05 versus control) (Fig. 1l and m).

Leukocortical lesions showed demyelination by LFB staining in the perivascular zone filled with infiltrating cells (Fig. 2c), but during late disease we also observed demyelinated leukocortical lesions completely devoid of infiltrating cells (Fig. 2e). We did not detect any demyelinated intracortical lesions, but most of the subpial lesions were demyelinated even during the acute disease phase (data not shown). As expected, many of the infiltrating cells around the leukocortical and intracortical lesions were positive for CD11b in EAE mice, while few perivenular CD11b positive cells were found in control mice (Fig. 2).

Neurofilaments in healthy myelinated axons are heavily phosphorylated and do not stain with SMI-32 antibodies (Sternberger and Sternberger, 1983), SMI-32 immunoreactivity has therefore been used as a marker of axonal damage in MS (Trapp et al., 1998; Peterson et al., 2001; Werner et al., 2001) and was abundant in the cortex of mice with EAE. Interestingly, the centre of the inflammatory lesions contained fewer SMI-32 positive axons than the border and the surrounding area (Fig. 2d and h). At higher magnification small SMI-32 positive ovoids (arrowheads) were found in close contact with activated microglia, and transected neural elements (arrows) could be detected around the cortical lesions (Fig. 2f'). A 3D-deconvolution from z-stacks of cortical layer V showed extended processes from microglial cells during acute disease highly associated with SMI-32 positive axons, while normal-appearing SMI-32 positive axons from control mice were not associated with microglia (Fig. 2i and j). We also observed satellitosis, a condition marked by an accumulation of glia cells around a neuron, in many of the cortical lesions (Fig. 2k and k'). Satellitosis is often a prelude to neuronophagia (phagocytosis of nerve cells), which could result in cell death (Song et al., 2006). These results suggest that microglia are closely associated with SMI-32 positive axons during disease, leading to generation of ovoids and transected axons.

Relapsing-remitting EAE results in persistent activation of microglia

Microglia are the resident immune effector cells of the CNS that become activated in response to injury with dramatic changes in their morphology as they become de-ramified with an enlarged cell body. Research has previously demonstrated that microglia in MS are activated even in areas outside the inflammatory lesions (Bjartmar et al., 2001). We found CD11b-positive microglia cells throughout the brain both in control and EAE that were highly abundant in the corpus callosum and cortex (Fig. 3 and data not shown). In order to investigate the change in activation of microglia cells at different clinical stages of disease, we performed high-resolution scanning of 30 individual microglia cells per time point and found that microglia in cortex were highly dynamic structures that were able to change their morphology after

S. Rasmussen et al.



Fig. 1 Immunization with PLP 139–15I results in generation of inflammatory lesions throughout the forebrain. SJL/J mice immunized with PLP 139–15I developed relapsing-remitting EAE. (a) Representative graph of the clinical course of one EAE mouse. Onset of clinical signs occurred at day II post immunization and peaked around day I7. EAE mice would then enter a remission phase followed by several relapses and remissions. The mice were sacrificed at various time points depending on the stage of disease progression. (b) Representative figure of inflammatory lesions (defined as a cluster of minimum 20 mononuclear cells) in the brain of an acute EAE mouse (H&E staining). (b') Higher magnification of montage in (b) (scale bar represents $250 \,\mu$ m). (c) The inflammatory lesions were found in various areas of the brain and were divided into the following three groups; (d) leukocortical, (e) intracortical and (f) subpial. There were also a number of purely white matter inflammatory lesions (g) (scale bar d-g represents $50 \,\mu$ m). (h-k) Scheme showing localization of inflammatory lesions from eight individual mice; control, acute, remission and relapse (red and green represents two separate mice). (l) The total number of inflammatory lesions per whole brain section was significantly increased at all timepoints compared with control (acute 34 ± 5 , P < 0.05; remission 22.5 ± 2.5 , P < 0.05; relapse 19 ± 4 , P < 0.05 versus control 3.5 ± 1.5 , n = 4 mice/group). (m) When divided into the three subgroups, the number of intracortical lesions (acute 7.5 ± 2.5 , P < 0.01; remission 4.5 ± 0.5 , P < 0.05; relapse 4.5 ± 0.5 , P < 0.01; remission 4.5 ± 0.5 , P < 0.05; relapse 4.5 ± 1.5 , P < 0.05; relapse 4.5

inflammatory stimuli. The microglia in control mice had the typical morphology of resting microglia (Stence *et al.*, 2001) with several very long and thin processes (Fig. 3a). However during acute disease the microglia became activated and developed an enlarged cell body with short thickened processes (Fig. 3b). During the first remission phase the microglia returned to a resting state morphology with longer and finer processes (Fig. 3c). Interestingly, after

Fig. 2 SJL mice develop cortical pathology during EAE. (**a**) Control mice show a fine pattern of myelination in the corpus callosum (LFB), (**b**) and show very little perivenular staining for SMI-32 (red) and CDIIb (green) (vessel is indicated with v). (**c**) Infiltrating cells and demyelination were found around a leukocortical vessel in EAE mice and (**d**) had a high infiltration of CDIIb microglia/macroghages (green) together with an increase in SMI-32 staining (red). (**e**) Leukocortial lesion with persistent demyelination (indicated with red arrowheads) during late relapse. (**f**) The leukocortical lesions were found at the border of the corpus callosum and the cortical grey matter centered on a vessel located in cortical layer VII. (**f**') A higher magnification of the lesion in (**d**) shows ovoids (arrows), where the axons were highly interacting with the inflammatory cells (scale bar represents 20 μm). (**g**) Control mice show no infiltrations in EAE mice have a high content of CDIIb microglia/macroghages (green) together with an increase SMI-32 staining (red). (**h**) Perivenular intracortical infiltrations in EAE mice have a high content of CDIIb microglia/macroghages (green) together with an increase SMI-32 staining (red) (vessel is indicated with v, scale bar in **b**, **d**, **g** and **h** represents 50 μm). 3D-deconvolution of z-stacks shows contact between CDIIb positive microglia/macrophages (green) and SMI-32 positive axons (red) around vessels in acute EAE (**j**) but not in control mice (**i**) (scale bar in **i** and **j** represents 10 μm). (**k**) Periventricular intracortical lesion (LFB) during acute EAE with (**k**') satellitosis showing infiltrating cells (red arrows) in very close contact with a neuron (N) (scale bar in **k** and **k**' represents 20 and 5 μm).



Control

Acute



Fig. 3 Persistent activation of microglia during EAE. (a) In the control cortex, the microglia had several long fine branches; (b) in acute disease the microglia had an enlarged cell body with several short, thickened processes. (c) In the first remission phase the microglia returned to a resting state with longer, finer branches. (d) In the late relapse, the microglia were re-activated and remained so in the late remission phase (e) (scale bar in a-e represents $20 \,\mu$ m). (f) The number of CDIIb-positive cells in a 2-mm block of the cortex was significantly increased throughout disease compared to control (acute 700 ± 58.63 , P = 0.0001; first remission 468.8 ± 59.84 , P = 0.0034; late relapse 600 ± 53.03 , P = 0.0002 and late remission 462.5 ± 78.06 , P = 0.0109 versus control 205 ± 26.69 , n = 3 mice/group). (g) Graph showing the average percentage of CD4+ cells in a CD45+ population in cortex. The percentage of CD4+ cells in cortex was significantly increased during acute disease (acute $6.27 \pm 0.2\%$ versus control $1.02 \pm 0.3\%$, P < 0.001, n = 3 mice/group). (h) The mean fluorescence intensity of CD45 in the total CDIIb population in cortex increases with time (Control, 173.0 ± 6.5 ; day 12, 195.0 ± 5.0 ; day 70, 181.5 ± 2.5 and day 170, 254.5 ± 14.5 , n = 3 mice/group).

the first relapse the microglia remained activated with an enlarged cell body and thicker shorter branches, with little change in their morphology even when the disease entered another remission or relapse (Fig. 3d and e). A similar change in morphology was observed for microglia in the corpus callosum (data not shown). The number of microglia in the corpus callosum and cortex was significantly increased during acute EAE and throughout the disease (Fig. 3f and data not shown). These data suggest that microglia become activated in acute EAE and may return to a resting state in the first remission, but with repeated bouts of inflammation they become persistently activated, a finding reminiscent of what has been described in chronic MS (Kutzelnigg *et al.*, 2005).

Persistent microglia activation is independent of T-cell infiltration

We enumerated infiltrating cells from the cortex and corpus callosum after dissecting these areas and isolating the cells at different time points during the disease. We found that the percentage of CD4 cells in cortex was significantly increased during acute disease compared to control $(6.27 \pm 0.2 \text{ versus } 1.02 \pm 0.3, P < 0.0001)$, however the percentage of CD4 cells decreased rapidly with time (Fig. 3g). CD4 and CD8 cells in the corpus callosum and CD8 cells in cortex also showed a transient increase in percentage during acute disease compared to controls followed by a steady decrease (data not shown). These results were confirmed by immunofluorescence staining for CD4 and CD8 in corpus callosum and cortex (data not shown). CD45 and CD11b has been suggested as markers for distinguishing between resting and activated microglia (Stevens et al., 2002), since resting microglia express low levels of CD45 and CD11b while activated microglia and macrophages express high levels of these surface markers. During acute disease (day 12), a population of highly activated CD45^{hi}, CD11b^{hi}cells (presumably macrophages) was readily apparent. This population decreased by day 70 and 170, but the resident microglia started to express higher levels of CD45, which was evident when looking at the mean fluorescence intensity (MFI) of CD45 for the total



Fig. 4 Immunized SJL mice develop lesions in cortical layers II/III, V and VII. (a) MECP2 immunohistochemistry shows localization of cortical layer II/III, V and VII (scale bar represents 100 μm). (b) In EAE, Cux-2 positive neurons in layer II/III, MeCP2 positive neurons in layer V and CTGF positive neurons in layer VII were found in the same cortical layers as those affected by clusters of activated CDIIb infiltrating cells. Direct interaction of microglia and the neuronal cell body is marked by white arrows on images shown as orthogonal-view. Cux-2, MeCP2 and CTGF positive neurons were also present in control mice, however, no microglia were found to be interacting with these cells (scale bar represents 25 μm, and 10 μm for orthogonal view).

CD11b population from both cortex (Fig. 3h) and corpus callosum (data not shown). In contrast, resting microglia express low levels of CD45 and CD11b. These results suggest that EAE results in a transient increase of infiltrating T cells in the forebrain which is in contrast to the persistent microglia activation.

Immunized SJL mice develop lesions in cortical layers II/III, V and VII

To examine which cortical layers were most affected by the infiltrating microglia we correlated the CD11b expression with different neuronal markers that are known to be expressed in specific cortical layers. Ctip2 is believed to play a critical role in the development of corticospinal motor neurons axonal projections (Arlotta et al., 2005; Molyneaux et al., 2005) and is used as a marker for cortical layer V. Cux-2 is expressed in layers II-IV of the cortex, and may have an important role in determining the neural fate of the upper cortical layers (Nieto et al., 2004). CTGF is only expressed on layer VII neurons, where it is thought to play an important regulatory role in modulating synaptic input to apical pyramidal neurons (Heuer et al., 2003), while MeCP2 may be involved in maturation and maintenance of neurons (Kishi and Macklis, 2004) and is expressed in neurons throughout the cortical layers. The expression of Cux-2, Ctip2, CTGF and MeCP2 was unaffected by the cortical pathology in EAE when compared to controls by measuring single cell pixel intensity (data not shown and Fig. 4). In control mice, resting microglia were detected throughout the cortex but did not show significant interaction with the neurons (Fig. 4). In contrast, during EAE clusters of activated microglia were found mostly associated with layers II/III, V and VII in close contact with the neurons and surrounding the neuronal cell body with their processes as shown by the 3D reconstruction of confocal imaging (Fig. 4). Activated microglia at 210 dpi were found to be very dynamic structures and their processes exhibited increased movement and interactions compared to the resting microglia when examined by time lapse confocal live imaging in brain slices (Supplementary Fig. 1 and Supplementary Movie 1).

Synaptic pathology in relapsing-remitting EAE correlates with the presence of activated microglia

Previous studies have demonstrated synaptic pathology with reduced immunoreactivity for synaptophysin in spinal cord of acute and chronic models of EAE (Zhu *et al.*, 2003; Marques *et al.*, 2006), and in leukocortical MS lesions (Wegner *et al.*, 2006). Synaptophysin is a synaptic protein localized on the membrane of synaptic vesicles (Zhu *et al.*, 2003) and it has been suggested that microglia in cortical

S. Rasmussen et al.



Fig. 5 Synaptic pathology in EAE correlates with close proximity of microglia. (a) Immunostaining with synaptophysin and CDIIb shows decreased staining for synaptophysin during EAE and highly increased CDIIb expression throughout the cortex (scale bar represents 15 μ m). (b) Synaptophysin immunostaining shows typical punctuated staining in the neuron cell body but not the nucleus (scale bar represents 5 μ m). (c) Quantification of the pixel intensity of synaptophysin in cortical neurons showed a significantly reduced signal in EAE mice compared to controls, also shown as a high-resolution quantitative 2.5-dimensional image of a representative neuron from control and EAE cortical layer V, maximal synaptophysin intensity is represented in red colour.

lesions target synaptic terminals, leading to functionally impaired neurons (Peterson et al., 2001). Alternatively, functionally impaired neurons exhibiting weak synaptophysin staining may trigger microglia activation. We assessed synaptophysin immunoreactivity qualitatively and quantitatively during acute EAE in relation to microglia activation. Synaptophysin immunostaining in control mice showed a punctate staining that encircled the neurons. In mice with acute disease, the staining was weak reflecting the low density of neuronal synapses in cortical layer II/III, V and VII correlating with high microglia content (Fig. 5a). Interestingly, normal-appearing synaptophysin positive neurons were not surrounded by microglia, but neurons with weak synaptic staining were closely associated with activated microglia (Fig. 5b). Quantification of the pixel intensity of synaptophysin shows a reduced signal in EAE compared to control (Fig. 5c). This suggests a relationship between activated cortical microglia and stripping of synaptic proteins in cortical neurons.

Since axonal transection and inflammation may cause neuronal death (Blinzinger and Kreutzberg, 1968; Peterson *et al.*, 2001), we evaluated the occurrence of apoptosis at various time points by a combination of TUNEL and neuronal immunocytochemistry. The number of TUNEL-positive cells was significantly increased during acute EAE (EAE 68.33 ± 8.4 versus control 2.67 ± 1.3 , P < 0.05, Supplementary Fig. 2a). Apoptosis was confirmed by activated caspase-3 staining (acute 118.0 ± 6.5 versus control 7.00 ± 1.5 , P < 0.001) (Supplementary Fig. 2b). However, among over one thousand TUNEL-positive cells, no TUNEL-positive neurons or activated caspase-3-positive neurons were detected at any of the time points investigated in EAE mice but instead the TUNEL-positive and the activated caspase-3-positive cells were in most cases found in close proximity to neurons (Supplementary Fig. 2c–e). These data suggest that in spite of cortical inflammation, axonal abnormalities and synaptic stripping, neuronal apoptosis does not play a role in the observed pathology.

Relapsing-remitting EAE results in severe demyelination of the corpus callosum

The corpus callosum plays a central role in interhemispheric communication and callosal atrophy in MS patients was shown to correlate with disability status (Martola *et al.*, 2006). In four separate experiments, LFB and MBP staining intensity was reduced in the corpus callosum in all EAE mice, and the integrity of the myelin fibers was compromised. Rarefaction of myelin was most severe in the medial part of the corpus callosum, adjacent to the lateral ventricle (LFB staining is shown in Fig. 6b and c) where the transparency of the pixel intensity of LFB



Fig. 6 Relapsing-remitting EAE results in demyelination of the corpus callosum. (a) Normal myelin staining in the corpus callosum in control mice, and a larger magnification (a') shows the fine branches of the radiating axons (arrows) extending out from the genu of the corpus calosum. (b and c) Rarefaction of myelin was observed in the corpus callosum of EAE mice during acute and the late relapse. (b' and c') This was associated with the disruption of corona radiata (arrows) (scale bar represent $100 \,\mu$ m). (d and e) Quantification of demyelination by measuring the transparency of LFB staining over a distance of $300 \,\mu$ m (white line) through the corpus callosum. There was a significant reduction of the myelin signal in acute EAE (acute 88.51 ± 0.5 versus control 20.69 ± 0.3 , P < 0.0001, n = 3 mice/group) seen as an increase in the transparency (d) and also in late relapse (late relapse 87.28 ± 0.7 versus control 20.69 ± 0.3 , P < 0.0001, n = 3 mice/group) (e).

was increased in acute EAE due to loss of signal (P < 0.0001 versus control, Fig. 6d) and in late relapse (P < 0.0001 versus control, Fig. 6e) consistent with loss of myelin. This was confirmed by myelin staining (Supplementary Fig. 3a-h) that also showed a significant decrease in the average MBP pixel intensity for all four EAE groups combined compared to the average control group (P < 0.0001, Supplementary Fig. 3m). The rarefaction of myelin was associated with a decreased staining of the radiating axons extending out from the genu of the corpus callosum (Fig. 6b' and c'). The severity of the white matter tissue damage was graded as 3 (the remaining fibres were disorganized and vacuoles were frequently observed) as previously described (Wakita et al., 1994). Since we found persistent demyelination throughout the corpus callosum and increased interaction of microglia in the cortex, we analysed the dynamics of microglia in the corpus callosum. We performed live imaging of brain slices of the corpus callosum during the chronic phase 210 dpi. We found that the microglia were increased in size with more elongated and highly dynamic processes, even after several months of chronic disease (Supplementary Fig. 4 and Movie 2). These behaviours were not seen in controls, suggesting that at the time of active demyelination and injury, the microglia persisted in a highly dynamic state that contrasted with the decreased T-cell presence in similar areas during the chronic phase (Fig. 3).

Decreased retrograde labelling of callosal projecting neurons in relapsing-remitting EAE

Focal lesions and diffuse axonal loss are common in MS. Post-mortem analysis of MS tissue showed more than 50% reduction of the total number of transcallosal axons compared to controls (Evangelou *et al.*, 2000). We injected the retrograde tracer hydroxystilbamidine into one hemisphere in order to examine cortical neurons contralateral to the injection (Mitchell and Macklis, 2005; Cheunsuang *et al.*, 2006). Cortical layers II/III, V and VII in mice with

2826 Brain (2007), **130**, 2816–2829

S. Rasmussen et al.



Fig. 7 Decreased retrograde labeling of callosal projecting neurons in relapsing–remitting EAE. (a) Images showing retrograde labeling with hydroxystilbamidine of callosal projection neurons contralateral to the injection site in control and EAE mice. Double staining with NeuN reveals a decrease in the number of double positive neurons in cortical layers II/III, V and VII in EAE when compared to control (scale bar represent $20 \,\mu$ m). (b–d) Percentage of NeuN positive neurons that are retrogradely labelled contralateral to the injection site. The percentage was significantly decreased in EAE compared with control both at 100 and 250 dpi in layer II/III (b) (EAEI00 29.94±1.859 versus control100 69.67±3.572, *P* < 0.001 and EAE250 12.69±2.136 versus control250 63.65±2.081, *P* < 0.001), in layer V (c) (EAEI00 20.00±1.981 versus control100 75.54±3.010, *P* < 0.001 and EAE250 11.63±1.211 versus control250 84.44±3.938, *P* < 0.001) and in layer VII (d) (EAEI00 15.75±1.961 versus control100 49.38±3.656, *P* < 0.001 and EAE250 16.44±2.934 versus control250 31.82±2.796, *P* < 0.001) (*n* = 2 mice/group).

late relapsing EAE had significantly fewer retrogradely labelled neurons compared with control mice (all had P < 0.001 versus control, Fig. 7). The loss of callosal projecting neurons correlated with a decrease in staining intensity of the radiating axons extending out from the genu of the corpus callosum (Fig. 8a) and a significant reduction in transcallosal axons (Fig. 8b and c). The pixel intensity of the hydroxystilbamidine staining in the corpus callosum was significantly reduced by 50% in EAE compared with control at 100 dpi (data not shown) and was persistent at 250 dpi $(53.19 \pm 0.3776 \text{ versus } 100.4 \pm 0.51,$ P < 0.0001) (Fig. 8d). These findings were consistent in two separate experiments done in a total of 12 mice. Since a decrease in retrogradely labelled neurons could be due to impaired axonal transport or to axonal transection, we measured axonal density at the genu of the corpus callosum by immunofluorescence staining of β-tubulin III and obtained the mean pixel intensity by confocal microscopy during acute and late relapse compared with controls. We found a 25% reduction in pixel intensity of transcallosal axons during the acute phase and 15% reduction during late relapse (data not shown). These data suggest that the decreased retrograde labeling of callosal projecting neurons can be attributed only partly to a loss of transcallosal axons but a significant impact is mediated by impaired axonal transport.

Discussion

Developing therapeutic strategies that target chronic progression in MS is an important and unfulfilled goal of MS treatment. For that goal to be achieved, we need to gain insight into the molecular pathology of MS progression and to model such therapeutic options experimentally (Compston, 2006; Gold et al., 2006). EAE is a useful animal model for MS but it is often criticized for the lack of correlation with MS pathology. Recent data in marmoset EAE has documented axonal pathology and cortical demyelination (Villoslada et al., 2000; Pomeroy et al., 2005; Merkler, Boscke et al., 2006), both of which are important aspects of MS pathology, however issues of feasibility and cost make the mouse model more suitable for studies of chronic progression than the marmoset model. Therefore an important question is to identify mouse strains susceptible to cortical pathology. EAE has been extensively studied in SJL mice but although it was reported that inflammatory infiltrates were present at all CNS anatomic levels, the focus has predominantly been on the spinal cord pathology (Brown and McFarlin, 1981; Sobel et al., 1990; Muller et al., 2000). Here we report that the SJL mouse immunized with PLP peptide 139-151 develops extensive forebrain pathology. We show cortical, periventricular subcortical and callosal lesions characterized

Brain (2007), **130**, 2816–2829 2827



Fig. 8 Decreased retrograde labelling of corpus callosum axons in relapsing-remitting EAE. (a) The staining of the radiating axons extending out from the genu of the corpus callosum and (b) the staining of the axons running through the corpus callosum was decreased in EAE compared to control (scale bar in **a** and **b** represents 5 μ m). (c) Quantification of axonal staining from callosal projection neurons by measuring the pixel intensity of hydroxystilbamidine over a distance of 200 μ m in the corpus callosum (red line) shows a significant reduction of the average hydroxystilbamidine signal in EAE compared with control at 250 dpi (EAE 53.19 \pm 0.3776 versus control 100.4 \pm 0.5127, P < 0.0001) (**d**) (n = 2 mice/group).

by demyelination, profound microglial activation and dysfunctional cortical neurons with axonal alterations in the corpus callosum.

Cortical lesions with demyelination and axonal alterations have been reported in MS (Peterson *et al.*, 2001; Kutzelnigg *et al.*, 2005) and subcortical dementia is a feature of the disease (Martola *et al.*, 2006). Brain atrophy is strongly associated with disease progression (Filippi *et al.*, 2003; Martola *et al.*, 2006), and may underlie potential alterations in projecting pathways. In this relapsing-remitting SJL model, we found profound functional alterations in specific cortical projecting neurons associated with chronic microglia activation. Microglia remain active during the chronic phase even during remission, in contrast to T-cell infiltration, which was limited to the acute phase. These data suggest that during the chronic phase of EAE inflammation is sustained by microglia, similar to the recent observations in MS tissue (Kutzelnigg et al., 2005). The microglia extend their processes around the cell bodies of neighbouring neurons, correlating with a loss of synaptic proteins. Although synaptic stripping may have a protective function in neurons (Trapp et al., 2007), the available data in EAE and other models of neurodegeneration suggest that it is deleterious (Zhu et al., 2003; Marques et al., 2006; Yoshiyama et al., 2007), moreover our retrograde labeling data suggest that axonal transport is altered in these projecting neurons in addition to the axonal loss in the corpus callosum.

We did not observe neuronal apoptosis in this study, but it is possible that rapid phagocytosis by microglia prevents the observation of apoptotic bodies and may become more apparent only when the neuronal apoptosis is increased (Li et al., 2003). However, alterations of synaptic function and axonal transport indicate that these neurons are dysfunctional, which is consistent with evidence that neuronal dysfunction is more prevalent that neuronal apoptosis in MS (Whitney et al., 1999; Black et al., 2000; Ibrahim et al., 2001; Lock et al., 2002; Ge, Gonen et al., 2004; Ge, Law et al., 2004; Lindberg et al., 2004; Jasperse et al., 2007; Phillips, 2007). Neuronal dysfunction characterized by alterations in gene expression of synaptic proteins and molecules important for neuronal stability, has been documented in normal-appearing white matter (NAWM) in MS (Lindberg et al., 2004) and EAE in the B6 MOG 35-55 model (Zhu et al., 2003), and in a rat model of EAE where alterations in gene expression in cerebral cortex, hippocampus and basal forebrain was associated with cognitive deficits (D'Intino et al., 2005). In our study the loss of retrograde labelling with preservation of NeuN-positive cells supports the concept of dysfunction, through axonal loss, axonal transection and decreased axonal transport.

Activated microglia appear to play a role in progression of neurological diseases (Kutzelnigg *et al.*, 2005; Yoshiyama *et al.*, 2007). Resting microglia survey the brain parenchyma, as shown by live imaging microscopy, and respond with directed migration to laser ablation injury (Nimmerjahn *et al.*, 2005). However, the dynamics of microglia during chronic injury are unclear. Here we show that during chronic EAE there is increased microglia surveillance and interaction with surrounding cells together with persistent activation. Inhibition of persistent microglia activation may prolong the life span of the mice in models of neurodegeneration (Heppner *et al.*, 2007; Adams *et al.*, 2007; Qin *et al.*, 2007; Yoshiyama *et al.*, 2007). Clearly a

2828 Brain (2007), **130**, 2816–2829

better understanding of the distinct phases and molecules that mediates microglia activation is needed since microglia may have a different function during the acute phase of activation than during chronic disease (Cardona et al., 2006). For example, ablation of early activated microglia worsens stroke suggesting that acute activation may have a protective role (Davalos et al., 2005). Through immunofluorescence and live imaging we have shown that activated microglia are not only found around inflammatory lesions but also in the normal appearing white and grey matter. Globally activated microglia have previously been demonstrated in NAWM for MS patients with axonal loss but preserved myelin (Bjartmar et al., 2001) and also in other animal models where the activated microglia were found far from the lesions (Yoshiyama et al., 2007). It is thought that small clusters of activated microglia precede the occurrence of inflammatory lesions. The mechanism of activation is presumably through diffusion of proinflammatory cytokines. Martiney et al. demonstrated that EAE could be suppressed by a macrophage-inactivating agent that inhibits the production of proinflammatory cytokines (Martiney et al., 1998).

An important goal in studying MS is to establish models that go beyond the modulation of T cells responses with new endpoints such as the preservation of synaptic proteins and axonal and neuronal function. Our observations demonstrate a novel alteration in neuronal projecting pathways in EAE that can help the study of molecular mechanisms of progression and evaluation of new strategies to stop neurodegeneration in MS.

Supplementary material

Supplementary material is available at Brain online.

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