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Phenotypic characterization of a *Csf1r* haploinsufficient mouse model of adult-onset leukodystrophy with axonal spheroids and pigmented glia (ALSP)

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Abstract

Mutations in the colony stimulating factor-1 receptor (*CSF1R*) that abrogate the expression of the affected allele or lead to the expression of mutant receptor chains devoid of kinase activity have been identified in both familial and sporadic cases of ALSP. To determine the validity of the *Csf1r* heterozygous mouse as a model of adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) we performed behavioral, radiologic, histopathologic, ultrastructural and cytokine expression studies of young and old *Csf1r*^{+/-} and control *Csf1r*^{+/+} mice. Six to 8-month old *Csf1r*^{+/-} mice exhibit cognitive deficits, and by 9-11 months develop sensorimotor deficits and in male mice, depression and anxiety-like behavior. MRIs of one year-old *Csf1r*^{+/-} mice reveal lateral ventricle enlargement and thinning of the corpus callosum. Ultrastructural analysis of the corpus callosum uncovers dysmyelinated axons as well as neurodegeneration, evidenced by the presence of axonal spheroids. Histopathological examination of 11-week-old mice reveals increased axonal and myelin staining in the cortex, increase of neuronal cell density in layer V and increase of microglial cell densities throughout the brain, suggesting that early developmental changes contribute to disease. By 10-months of age, the neuronal cell density normalizes, oligodendrocyte precursor cells increase in layers II-III and V and microglial densities

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remain elevated without an increase in astrocytes. Also, the age-dependent increase in CSF-1R+ neurons in cortical layer V is reduced. Moreover, the expression of *Csf2*, *Csf3*, *Il27* and *Il6* family cytokines is increased, consistent with microglia-mediated inflammation. These results demonstrate that the inactivation of one *Csf1r* allele is sufficient to cause an ALSP-like disease in mice. The *Csf1r*^{+/-} mouse is a model of ALSP that will allow the critical events for disease development to be determined and permit rapid evaluation of therapeutic approaches. Furthermore, our results suggest that aberrant activation of microglia in *Csf1r*^{+/-} mice may play a central role in ALSP pathology.

Keywords

Leukodystrophy; ALSP; HDLS; CSF-1R; microglia; dysmyelination; GM-CSF

INTRODUCTION

Hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS) (Axelsson et al., 1984) and familial pigmentary orthochromatic leukodystrophy (POLD) (Van Bogaert and Nyssen, 1936) are similar (Marotti et al., 2004; Wider et al., 2009), rare, autosomal dominant, neurodegenerative disorders characterized by adult-onset dementia with motor impairment and epilepsy. Due to their similarities and the demonstration that both diseases were caused by inactivating mutations in the region encoding the intracellular kinase domain of the colony stimulating factor-1 receptor gene (*CSF1R*) (Nicholson et al., 2013; Rademakers et al., 2011), it was suggested these two diseases be collectively renamed adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) (Nicholson et al., 2013; Wider et al., 2009).

The CSF-1R is regulated by two cognate ligands, CSF-1 (Dai et al., 2002; Stanley and Heard, 1977) and interleukin-34 (IL-34) (Lin et al., 2008; Wei et al., 2010) and is expressed on microglia (Sawada et al., 1990), neural progenitor cells (NPCs) and several neuronal subtypes (Luo et al., 2013; Nandi et al., 2012; Wang et al., 1999). It is required for the development and maintenance of all microglia (Ginhoux et al., 2010). CSF-1 or IL-34 activation of the CSF-1R on NPCs suppresses NPC self-renewal and stimulates neuronal survival and differentiation (Nandi et al., 2012). Furthermore, brains of *Csf1r*^{-/-} nullizygous mice have gross anatomical and histological abnormalities (Erblich et al., 2011; Nandi et al., 2012) that affect areas (cortex, corpus callosum) disrupted in ALSP (Axelsson et al., 1984; Kinoshita et al., 2014; Konno et al., 2014; Rademakers et al., 2011; Schiffmann and van der Knaap, 2009; Wider et al., 2009).

The *CSF1R* mutations first described in HDLS families, included missense mutations affecting highly conserved residues and splice-site mutations leading to in-frame deletions. Furthermore, the discovery of a HDLS patient with a *CSF1R* frame-shift mutation that abolished protein expression proved that *CSF1R* haploinsufficiency is sufficient to cause ALSP (Konno et al., 2014).

Since the initial report of inactivating mutations in man (Rademakers et al., 2011) could be explained by haploinsufficiency, we studied *Csf1r*^{+/-} mice. Here we show that these mice

exhibit behavioral, radiologic, histopathologic and ultrastructural alterations associated with neuronal degeneration and microgliosis, similar to the changes observed in ALSP patients.

MATERIAL AND METHODS

Mouse models, breeding and analyses

The generation, maintenance and genotyping of *Csf1r*^{+/-} mice has been described previously (Dai et al., 2002). These mice are not osteopetrotic (Dai et al., 2004). Mice were backcrossed for more than 10 generations onto the C57/BL6 background and littermate *Csf1r*^{+/+} mice were used as controls. The behavioral studies (6-11 months of age) involved 21 *Csf1r*^{+/-} (11 females, 10 males) and 18 sex/age-matched control *Csf1r*^{+/+} mice (7 females, 11 males). Histopathology, cytokine and ultrastructural studies were carried out at 10-12 months of age utilizing a subgroup of 10 male and female *Csf1r*^{+/-} mice and their controls selected based on poor motor coordination and increased anxiety-like behaviors. The motor coordination and anxiety scores of this group were significantly different from the group of 19 *Csf1r*^{+/+} mice ($p=0.0003$ and $p=0.037$, respectively). The remaining 10 *Csf1r*^{+/-} mice were not significantly different from the *Csf1r*^{+/+} mice ($p>0.05$) in these parameters, indicating incomplete penetrance of the symptoms by 9-11 months of age. Additional *Csf1r*^{+/-} and littermate control *Csf1r*^{+/+} mice were subject to histopathologic analysis at 11-weeks of age.

Behavioral studies

To assess cognition, we examined recognition memory (novel object recognition) and visuospatial memory (novel object placement) (Ennaceur and Delacour, 1988) tests, analogous to assessments conducted in humans (Caterini et al., 2002; Lawrence et al., 2000). Motor coordination was assessed as the number of slips made while crossing a round, wooden balance beam (Gulinello et al., 2008; Stanley et al., 2005). Depression-like behavior was assessed as immobility, using the Porsolt Forced Swim Test (Porsolt et al., 1977a; Porsolt et al., 1977b). Anxiety-like behavior was assessed in an elevated plus maze with 2 open and 2 closed arms, in which greater exploration of the open arms indicates lower levels of anxiety-like behavior (Pellow et al., 1985). Olfaction was examined using a standard buried food test (Erblich et al., 2011). Data from the novel object location test were analyzed with 2-way ANOVA (sex by genotype) (preference score) or chi square (preference category). All other tests were analyzed with either a 2 way ANOVA (sex by genotype) or a 2-way repeated measures ANOVA (sex by genotype by age), followed by pairwise comparisons where appropriate.

MRI imaging

Mice were imaged on an Agilent Direct Drive 9.4 T MRI system (Agilent Technologies, Santa Clara, CA) using 60 gauss/cm imaging gradients with 180 μ s rise times. Mice were anesthetized with 1.5% isoflurane in room air, and respiratory rate and oxygenation saturation were monitored and maintained within normal ranges, while body temperature was maintained at 39°C, using a warm air circulator (SA instruments, Bayshore, NY). A 1.8 mm actively decoupled surface coil (Doty Scientific, Columbia, SC) was used for acquisition, and a 60 mm ID birdcage volume coil (M2M Imaging, Cleveland, OH) was

used for radio frequency transmission. Diffusion tensor imaging was accomplished using 30 directions of diffusion encoding (Jones and Leemans, 2011), 18 mm FOV, 18 slices with 0.6 mm thickness (10% gap) with matrix 128×128 . Echo Planar Imaging was used to acquire the data, employing a 6-shot interleaved spin echo acquisition with 260 kHz bandwidth. Fractional anisotropy (FA) images were calculated using the DTI data set within the FSL (FMRIB, Oxford, UK) image-processing package. Lateral ventricle volumes and cortical and callosal thicknesses were measured using MIPAV 7.1.1 freeware (mipav.cit.nih.gov).

Ultrastructural studies

Mice were perfused with 30 ml of cold phosphate buffered saline (PBS) containing 10U heparin/ml followed by 30 ml of 2% paraformaldehyde (PFA) in PBS. The brains were dissected, sliced into 2 mm thick slices and placed in cacodylate fixation buffer (2% PFA, 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4) for 40 min at 20°C. Corpus callosum was dissected, incubated overnight at 4°C in cacodylate fixation buffer, embedded, sectioned, stained and examined by transmission electron microscopy using a JEOL 1200EX microscope.

Immunofluorescence staining of brain sections

Mice were perfused with 20 ml cold PBS containing 10U heparin/ml followed by 30 ml of 4% PFA in PBS. The brains were removed from the skull, incubated successively with 4% PFA in PBS (16h, 4°C) and 20% sucrose in PBS (16h, 4°C), embedded in Shandon M1 embedding matrix (Thermo Scientific, Waltham, MA) and cut into 30 μ m sections. Floating sections were stained using the antibodies to phosphorylated neurofilament heavy protein epitope (NFHP) (mouse IgG1; Covance, New York, NY), myelin basic protein (MBP) (mouse IgG2b; AbCam, Cambridge, MA), ionized calcium binding adaptor molecule 1 (Iba1) (rabbit IgG; Wako Chemicals, Richmond, VA), platelet-derived growth factor receptor- α (PDGFR α) (goat IgG; R&D Systems, Minneapolis, MN), NeuN (mouse IgG1; Millipore, Billerica, MA), glial fibrillary acidic protein (GFAP) (mouse IgG2b; BD, Franklin Lakes, New Jersey) and CSF-1R (IK) (rabbit IgG; produced in our laboratory). Secondary antibodies, conjugated to either Alexa 488, Alexa 594 or Alexa 647, were from Life Technologies (Grand Island, NY). Images were captured using an Olympus Bx51 upright fluorescence microscope with Olympus MicroSuite Five Biological software. Quantification of cell numbers was performed manually. Areas were measured using Image J software (imagej.net). Images were cropped and adjusted for brightness, contrast and color balance using Adobe Photoshop CS4.

RNA isolation and QRT-PCR profiling

RNA was isolated from 2 mm thick sections of the anterior primary motor cortex (M1) and corpus callosum (CC) using the RNeasy mini kit (Qiagen, Gaithersburg, MD) according to manufacturer's instructions. Reverse transcription was carried out using 160 ng of total RNA and the RT² First Strand Kit (Qiagen). RT-PCR reactions were carried out using the RT² Profiler™ PCR Array Mouse Inflammatory Cytokines & Receptors (Qiagen) in an Eppendorf Mastercycler ep realplex 2 thermal cycler.

Measurements of brain CSF-1 and IL-34

Brains were isolated, sliced into 2 mm thick coronal sections and the indicated regions dissected and frozen at -80°C . Proteins were extracted by homogenizing the tissue in ice-cold lysis buffer (0.1% NP-40, 10 mM Tris-HCl, 50 mM NaCl, 30 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50 mM NaF, 100 μM Na_3VO_4 , 5 μM ZnCl_2 , 1 mM benzamide, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin, pH 7.2) at 4°C . The lysates were centrifuged for 15 min at $13,000 \times g$ at 4°C . Supernatants containing 70 μg of total protein were used to determine the concentration of CSF-1 and IL-34 using ELISA kits from Ray Biotech Inc. (Norcross, GA) and BioLegend (San Diego, CA), respectively.

Protein isolation and western blot analysis

Proteins were extracted from 2 mm thick sections of the anterior M1 and CC by homogenization in lysis buffer as indicated above. Aliquots containing 30 μg of total protein were resolved by SDS-PAGE and specific protein expression examined by western blotting. The chemiluminescence signal was detected using a Fujifilm LAS3000 imager (Fujifilm, Valhalla, NY) and quantified by the use of MultiGauge software (Fuji).

RESULTS

Adult *Csf1r*^{+/-} mice exhibit cognitive, emotional, sensorimotor and olfactory deficits

ALSP patients initially present with memory loss, apathy, depression and repetitive behaviors, as well as cognitive and sensorimotor symptoms (Axelsson et al., 1984; Freeman et al., 2009; Mitsui et al., 2012; Sundal et al., 2013; van der Knaap et al., 2000). Cognitive assessment revealed significantly impaired visuospatial memory in *Csf1r*^{+/-} mice, compared with *Csf1r*^{+/+} control mice, that was evident as early as 6-8-months of age (Fig. 1A). The memory deficits were not confounded by lack of object exploration (Fig. 1B), altered levels of ambulation or exploration in the open field (Fig. 1C). By 9-11 months of age, *Csf1r*^{+/-} mice developed significant sensorimotor defects compared with *Csf1r*^{+/+} control mice (Fig. 1D). Two of the *Csf1r*^{+/-} females became ataxic at 8.5 and 11 months of age. By 9-11 months, males, but not females, had significant depression (Fig. 1E) and anxiety-like (Fig. 1F) behaviors.

Csf1r-deficient *Csf1r*^{-/-} mice exhibit olfactory bulb atrophy (Nandi et al., 2012) and olfactory deficiencies have been observed in neurodegenerative diseases (Wszolek and Markopoulou, 1998). *Csf1r*^{+/-} mice of both sexes were found to have significant olfactory deficits compared with wild-type control mice at 9-11 months of age (Fig. 1G).

MRI analysis of 12 month-old *Csf1r*^{+/-} mice demonstrates callosal thinning and increased lateral ventricle volumes

ALSP is characterized by cerebral white matter lesions, predominantly involving the frontal and parietal white matter, becoming confluent and symmetrical with disease progression, with thinning of the corpus callosum, increased lateral ventricle (LV) size and evolving cortical atrophy (Axelsson et al., 1984; Freeman et al., 2009; Kondo et al., 2013; Konno et al., 2014; Sundal et al., 2012; Van Gerpen et al., 2008)³². *In vivo* MRI analysis of a 12-month old male *Csf1r*^{+/-} mouse with severe sensorimotor dysfunction showed cerebral

white matter lesions, thinning of the corpus callosum and increased LV size as compared to the *Csf1r*^{+/+} control (Fig. 2A, B). The corresponding FA images suggest dysmyelination of the cortical and central callosal region in this mouse (Fig. 2B). In contrast, apart from decreased cortical FA and a small increase in LV size, these changes were largely absent in images obtained from a *Csf1r*^{+/-} male with mild sensorimotor dysfunction (Fig. 2A, B). Quantification of LV volume, cortical and callosal thickness for 3 *Csf1r*^{+/+} and 7 *Csf1r*^{+/-} mice indicated significant changes in LV volume and callosal thickness in severely affected *Csf1r*^{+/-} mice (Fig. 2C, D).

Abnormal myelination and neuronal degeneration of white matter tracts in adult *Csf1r*^{+/-} mice

Thinning of the corpus callosum (Kinoshita et al., 2014; Kondo et al., 2013; Konno et al., 2014) that may precede disease onset by up to 5 years (Konno et al., 2014), loss of myelin sheaths and axons and the presence of axonal spheroids, have been reported in ALSP patients (Axelsson et al., 1984; Freeman et al., 2009; Konno et al., 2014; Van Gerpen et al., 2008). We therefore examined the morphology of the corpus callosum by electron microscopy. Ultrastructural abnormalities found in *Csf1r*^{+/-} mice included the presence of hypermyelinated axons and axonal spheroids as well as an increase in demyelinated axons (Fig. 3).

Early changes in myelination and neuronal and glial cell populations suggest developmental abnormalities in *Csf1r*^{+/-} brains

There were no gross abnormalities of brain size, weight or olfactory bulb development in *Csf1r*^{+/-} mice (data not shown). However, staining for NFHP and MBP indicated that there was increased axonal and myelin staining in the upper cortical layers of 11-week old *Csf1r*^{+/-} forebrains and increased MBP staining in 10-month old *Csf1r*^{+/-} brains compared with their wild-type controls (Fig. 4A, B). Interestingly, while the number of NeuN⁺ mature neurons did not differ in any cortical layer at 10-months of age (Fig. 4C), there was an increase in the PDGFR α ⁺ oligodendrocyte precursors (OLPs) in layers II-III and V of the *Csf1r*^{+/-} brains (Fig. 4D). OLP proliferation, differentiation and oligodendrocyte myelinating activity are regulated by factors secreted by microglia and astrocytes (Miron et al., 2013; Nakanishi et al., 2007). Whereas staining for GFAP showed no change in astrocyte densities (Fig. 5), Iba1 staining revealed an increase in microglial density throughout the brains of *Csf1r*^{+/-} mice (Fig. 6). Of additional interest, a significant proportion of the mature neurons in cortical layer V were lost between 11-weeks and 10-months of age in the *Csf1r*^{+/-} mice (Fig. 4C), suggesting selective neuronal loss in this area. As callosal neurons are primarily derived from layer III and upper layer V (Ivy and Killackey, 1981), this reduction could contribute to the callosal axonal abnormalities and the callosal thinning detected at 12 months (Fig. 2D) as disease progresses.

The age-dependent increase in CSF-1R-expressing layer V cortical neurons is suppressed in *Csf1r*^{+/-} mice

The CSF-1R is expressed on microglia (Sawada et al., 1990), NPCs and some neuronal subtypes, but is absent from oligodendrocytes (Luo et al., 2013; Nandi et al., 2012; Wang et

al., 1999). Activation of the CSF-1R on NPCs suppresses their self-renewal and stimulates neuronal survival and differentiation (Nandi et al., 2012). Furthermore, upregulation of the CSF-1R in injured neurons is neuroprotective (Luo et al., 2013). We therefore compared the density of CSF-1R+ neuronal cells in 11-week and 10-month-old *Csf1r*^{+/+} and *Csf1r*^{+/-} mice in different cortical layers. Whereas the percent of layer V and VI neurons expressing the CSF-1R increased with age in both *Csf1r*^{+/+} and *Csf1r*^{+/-} mice, this increase was significantly reduced in layer V of *Csf1r*^{+/-} mice (Fig. 7). Thus insufficient expression of the CSF-1R in a fraction of layer V neurons of aging *Csf1r*^{+/-} mice (Fig. 7) may explain the neuronal cell loss in this layer (Fig. 4C). In addition, as CSF-1R and its ligands are highly expressed in this region of the cortex during early postnatal development (Nandi et al., 2012), *Csf1r* haploinsufficiency may upset the normal regulation of neurogenesis in layer V neurons.

Reduced expression of the CSF-1R without compensatory increases in CSF-1R ligand expression in *Csf1r*^{+/-} brains

To establish that *Csf1r* haploinsufficiency results in reduced CSF-1R expression, we determined the levels of CSF-1R mRNA and protein expression. Expression of both CSF-1R mRNA (Fig. 8A) and the mature ~165-kDa CSF-1R that is expressed on the cell surface (Fig. 8B, C) were reduced by ~50% in *Csf1r*^{+/-} brains at 12 months. It is not clear why expression of the mature CSF-1R is differentially affected. However, a similar situation has been observed with dominant inactivating missense mutations in the *Csf1r* that become trapped in the Golgi as the ~130-kDa precursor (Hiyoshi et al., 2013) and may reflect loss of feedback control from the active, mature CSF-1R on its own trafficking.

As shown with CSF-1R inhibition (Miron et al., 2013), *Csf1r* haploinsufficiency could result in a compensatory increase in the levels of either of the two CSF-1R ligands. This could cause prolonged signaling through residual CSF-1Rs and the increase in microglial densities in *Csf1r*^{+/-} brains (Fig. 6). Although, as expected, IL-34 levels were higher than CSF-1 levels (Wei et al., 2010), there were no significant differences between *Csf1r*^{+/+} and *Csf1r*^{+/-} mice in mRNA or protein expression of these ligands in various brain regions (Fig. 8D).

Gene expression patterns in adult *Csf1r*^{+/-} brains suggest a pro-inflammatory phenotype of microglia

Because the increase of microglia in *Csf1r*^{+/-} brains might reflect reactive microgliosis, we determined the expression of mRNAs of known markers of inflammation in the frontal cortex and corpus callosum of *Csf1r*^{+/+} and *Csf1r*^{+/-} mice by QRT-PCR (Fig. 8E, F). In 12 month-old *Csf1r*^{+/-} brains, levels of mRNAs encoding the growth factors, granulocyte macrophage-CSF (GM-CSF, *Csf2*) and granulocyte-CSF (G-CSF, *Csf3*), were elevated. In addition, the expressions of the GM-CSF target genes, interleukin-6 (IL-6), IL-27 and Fas ligand (*Fasl*) (Kosloski et al., 2013; Suzumura et al., 1996; Xiao et al., 2002) were altered. Furthermore, the expression of IL-6 family ligands and receptors, leukemia inhibitory factor (LIF) and IL-6R α , involved in OLP proliferation and maturation, were elevated in 12-month-old *Csf1r*^{+/-} mouse brains (Fig. 8F). In contrast, the mRNA levels of only two cytokines, GM-CSF and G-CSF, were elevated in 7 week-old *Csf1r*^{+/-} mouse brains (Fig. 8E).

DISCUSSION

Since the first descriptions of POLD in 1936 (Van Bogaert and Nyssen, 1936) and of HDLS in 1984 (Axelsson et al., 1984), over 100 ALSP patients with quite variable clinical presentations have been reported. The median age of onset is 42 ± 13 years (range 8-78) with a disease duration of 5 ± 7 (range 1-34) years that is unrelated to the time of onset (Ahmed et al., 2013; Freeman et al., 2009; Guerreiro et al., 2013; Hoffmann et al., 2014; Karle et al., 2013; Kinoshita et al., 2014; Kleinfeld et al., 2013; Kondo et al., 2013; Konno et al., 2014; Mitsui et al., 2012; Rademakers et al., 2011; Sundal et al., 2012; Van Gerpen et al., 2008; Wider et al., 2009). Symptoms may vary according to gender (Hoffmann et al., 2014), and clinical presentation is variable. Patients often present with sensorimotor and neuropsychiatric symptoms, including depression, behavioral changes, spastic paraplegia, dementia and seizures, leading to frequent and diverse clinical misdiagnoses. The initial symptoms progress to dementia and death (Axelsson et al., 1984; Guerreiro et al., 2013; Marotti et al., 2004; Sundal et al., 2012; Wider et al., 2009). By MRI, ALSP is characterized by patchy cerebral white matter lesions, often initially asymmetrical, but becoming confluent and symmetrical with disease progression (Freeman et al., 2009; Konno et al., 2014; Sundal et al., 2012; Van Gerpen et al., 2008). The changes predominantly involve the frontal and parietal white matter, with thinning of the corpus callosum and increased LV size being early features (Kondo et al., 2013; Konno et al., 2014).

Our results indicate that *Csf1r*^{+/-} mice exhibit behavioral, radiologic and histopathologic changes similar to those seen in ALSP patients. By 8 months of age, *Csf1r*^{+/-} mice have developed cognitive defects and by 10 months of age approximately 50% of the mice have sensorimotor defects, depression and anxiety-like behaviors characteristic of early ALSP in man, as well as olfactory deficiencies observed in other neurodegenerative diseases (Wszolek and Markopoulou, 1998). The sensorimotor defects are correlated with significant histopathologic changes in the motor cortex. MRI revealed callosal thinning and increased LV volumes, as reported for patients with early stage ALSP. Histopathology indicated abnormal myelination and neurodegeneration and increased microglial density, also reported for ALSP (Axelsson et al., 1984; Freeman et al., 2009; Kinoshita et al., 2014; Kondo et al., 2013; Konno et al., 2014; Van Gerpen et al., 2008). Together, these findings indicate that the *Csf1r*^{+/-} mouse is a useful model of ALSP.

We observed increased myelination in pre-symptomatic 11 week-old *Csf1r*^{+/-} mice that was still apparent in 10 month-old mice. Increased myelination has not been reported in ALSP patient brains, possibly because the histopathological evaluations are predominantly post-mortem, when neurodegeneration is extensive. Other studies have shown that hypermyelination may trigger neuronal degeneration, expansion of microglia and leukodystrophy in mice (Jaini et al., 2013; Maire et al., 2014). Furthermore, by increasing autoantigenic load, hypermyelination exacerbates CNS autoimmunity (Jaini et al., 2013). Thus, the hypermyelination in *Csf1r*^{+/-} mice may contribute to neuronal loss.

We suspect that the hypermyelination is due to the presence of oligodendrocytes generated from the increased numbers of OLPs. Although oligodendrocytes do not express the CSF-1R (Erblich et al., 2011; Nandi et al., 2012), they express the GM-CSF receptor and GM-CSF

stimulates oligodendrocyte proliferation (Baldwin et al., 1993). Furthermore, GM-CSF and G-CSF promote neuronal differentiation (Kruger et al., 2007). Thus elevation of GM-CSF in the brains of young *Csf1r*^{+/-} mice (Fig. 8E) could explain their increased axonal and myelin complement (Fig. 4A, B). GM-CSF is also mitogenic for microglia, and we observed increased microglial densities throughout the brains of these mice (Fig. 6). Although the source of GM-CSF in the *Csf1r*^{+/-} brain remains to be identified, GM-CSF is constitutively expressed by CNS neurons (Dame et al., 2002), microvascular endothelium, smooth muscle pericytes (Hart et al., 1992), and following activation, by astrocytes (Henze et al., 2005) and microglia (Gabrusiewicz et al., 2011). Reduced *Csf1r* expression in neurons or microglia may, directly or indirectly, stimulate production of GM-CSF.

Consistent with results in young mice, analysis of cytokine expression in 1 year-old *Csf1r*^{+/-} mice revealed continued elevation of GM-CSF and G-CSF. GM-CSF decreases the expression of Fas ligand in microglia, thereby prolonging the microglial lifespan and induces expression of IL-6 and IL-27 (Kosloski et al., 2013; Suzumura et al., 1996; Xiao et al., 2002). The genes encoding these cytokines were correspondingly regulated in *Csf1r*^{+/-} brains, suggesting that GM-CSF signaling in microglia was activated. GM-CSF is a known activator of microglia, increasing their ability to engage in phagocytosis of unopsonized myelin (Smith, 1993), to produce IL-6 and to present antigen in the absence of additional inflammatory stimulation (Fischer et al., 1993). The gene expression for IL-6 receptors (*Il6ra*) was also increased in the brains of the older *Csf1r*^{+/-} mice. Concomitant upregulation of IL-6 and IL-6R α in the brain may enhance IL-6 trans-signaling, a process that involves the binding of the soluble IL6R α /IL6 complex to gp130 to mediate IL-6 neurotoxicity (Campbell et al., 2014). Thus GM-CSF-activated microglia could trigger neurodegeneration via several mechanisms (Fig. 9).

On the basis of our results, we speculate that elevation of GM-CSF through its action on oligodendrocytes, microglia and NPC plays an important role in the pathology of ALSP. Thus inhibitors of GM-CSF may be therapeutically important. Genetic and pharmacological approaches, using the ALSP mouse model, can be used to investigate the contributions of GM-CSF signaling to disease development. It is unclear whether the early microgliosis we observe has an active (triggering) or reactive contribution to disease pathology, a question that may be resolved by characterization of microglial lineage-specific *Csf1r* haploinsufficient mice. In addition, anomalies in the cortical neurons in layer V, where the CSF-1R and IL-34 are highly expressed during development (Nandi et al., 2013; Nandi et al., 2012), suggest that loss of a single allele of the *Csf1r* may directly disrupt neurogenesis. Neuronal development in *Csf1r*^{+/-} mice can be studied in detail and the contribution of neuronal lineage-specific *Csf1r* haploinsufficiency to disease development assessed. Thus the existence of this ALSP mouse model should be invaluable for understanding the basis of disease and in developing novel therapies.

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Abbreviations

ALSP	adult-onset leukoencephalopathy with axonal spheroids and pigmented glia
CSF-1R	colony stimulating factor-1 receptor
FA	fractional anisotropy
GFAP	glial fibrillary acidic protein
G-CSF	granulocyte-CSF
GM-CSF	granulocyte macrophage-CSF
HDLS	hereditary diffuse leukoencephalopathy with axonal spheroids
Iba1	ionized calcium binding adaptor molecule 1
IL-34	interleukin-34
LIF	leukemia inhibitory factor
LV	lateral ventricle
MBP	myelin basic protein
NFHP	neurofilament high molecular weight phosphorylated epitope
NPC	neural progenitor cells
OLP	oligodendrocyte precursors
PDGFRα	platelet-derived growth factor receptor α
PFA	paraformaldehyde
PBS	phosphate buffered saline
POLD	pigmented orthochromatic leukodystrophy.

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Highlights

- Validation of the *Csf1r*^{+/-} mouse as the first animal model of ALSP
- *Csf1r*^{+/-} mice develop cognitive deficits followed by sensorimotor dysfunction
- *Csf1r*^{+/-} mouse brains show enlarged lateral ventricles and callosal thinning
- *Csf1r*^{+/-} mice show dysmyelination, neurodegeneration and microgliosis
- Low CSF-1R signaling and high GM-CSF levels may trigger the microglial activation

Figure 1. Older adult *Csflr*^{+/-} mice exhibit cognitive, sensorimotor, emotional and olfactory deficits

(A) Lower preference for the novel (re-located) object in 6-8 month-old *Csflr*^{+/-} compared with *Csflr*^{+/+} mice, indicative of visuospatial memory deficits (left panel; *, $F_{1,35}=4.9$; $p<0.03$) and failure of a higher percentage of *Csflr*^{+/-} mice in the novel object location test (defined as <55%, Chi Square $p<0.01$; right panel). (B) Object exploration during the object location test and (C) ambulation in the open field at 6-8 months of age. (D) Motor coordination deficits of 9-10 month-old *Csflr*^{+/-} mice, assessed by the number of slips on a narrow round beam (*, $F_{1,37}=7.8$; $p<0.008$). (E) Increased depression-like behavior in 9-11 month-old *Csflr*^{+/-} male mice, assessed as immobility in the forced swim test (*, $F_{1,20}=8.2$; $P<0.01$). (F) Increased anxiety-like behavior in 9-11 month-old *Csflr*^{+/-} male mice (elevated plus maze; * $F_{1,20}=9.4$; $P<0.005$). (G) Olfactory deficits in 9-11 month-old *Csflr*^{+/-} mice (buried food test; *, $F_{1,34}=6.3$, $P<0.02$). All mice readily consumed palatable food in a visible food trial (not shown). Mouse numbers are indicated within the bars. The effect of sex was included as a factor in all tests. If sex was not significant, sexes were combined in analyses and graphs. If sex was a significant main effect, the graphs and analyses show the sexes individually.

Figure 2. MRI changes, abnormal myelination and neuronal degeneration of white matter tracts in *Csf1r*^{+/-} brains

(A) Top row: Selected slices from reconstructed diffusion tensor imaging maps of brains of 12-month-old *Csf1r*^{+/-} mice with either mild (13-18 balance beam slips) or severe (24-39 slips) sensorimotor deficits and of control *Csf1r*^{+/+} mice (3-9 slips). (B) FA maps corresponding to the first and third panels from the left in (A). Areas of low FA in *Csf1r*^{+/-} compared to control *Csf1r*^{+/+} mice are outlined with dotted lines. (C) Quantification of lateral ventricle volumes relative to *Csf1r*^{+/+} control volumes derived by analysis of slices starting at +1.18 mm and ending at -1.82 mm from the bregma. LV volume was increased in *Csf1r*^{+/-} compared to *Csf1r*^{+/+} control mice ($F_{2,7} = 8.018$, $p = 0.0014$, Kruskal-Wallis test). (D) Quantification of cortical (Cx) and corpus callosum (CC) thicknesses in slices at +1.18 mm from the bregma. Compared to *Csf1r*^{+/+} mice, *Csf1r*^{+/-} mice exhibited decreased callosal thickness ($F_{2,7} = 7.634$, $p = 0.0048$), whereas Cx thickness was not significantly changed ($F_{2,7} = 2.88$; $p = 0.26$) ($n = 3$ mice per group). The p-values for the difference between the *Csf1r*^{+/-} severe and *Csf1r*^{+/+} control groups in LV volume (C) and callosal thickness (D) were determined using Dunn's post-hoc analysis.

Figure 3. Dysmyelination and neurodegeneration in the white matter of *Csf1r*^{+/-} mice
 Transmission electron microscopy images showing the morphology of corpus callosum neurons obtained from 10-month-old *Csf1r*^{+/+} (A) and *Csf1r*^{+/-} (B, C) mice. Arrows in (B) indicate hypermyelinated axons and arrowheads indicate non-myelinated axons. The arrow in (C) indicates an axonal spheroid. Scale bars: 500 nm (A, B); 1 μm (C).

Figure 4. Changes in myelination and neuronal and oligodendrocyte precursor cell populations in the primary motor cortex of *Csf1r*^{+/-} male mice

(A) Immunostaining for mature neurofilaments (NFHP) and myelin basic protein (MBP) in coronal brain sections. Scale bar: 1 mm. (B) Higher magnification of the boxed areas shown in (A) illustrating the increased MBP and NFHP staining in the upper cortical layers at 11 weeks of age and the increased MBP staining at 10 months of age. Scale bar: 125 μ m. (C) Quantification of NeuN⁺ neurons in individual cortical layers. Note that the increase in cortical layer V neurons of *Csf1r*^{+/-} mice relative to *Csf1r*^{+/+} controls at 11 weeks (w) is lost by 10 months (m). (D) PDGFR α ⁺ OLPs are increased in the cortical layers II-III and V of 10 month-old *Csf1r*^{+/-} mice. Means $\pm$ S.E.M.; n=3; * p<0.05 compared to *Csf1r*^{+/+} controls, Student's t test.

Figure 5. Normal astrocyte density in brains of 10 month-old *Csf1r*^{+/-} male mice
 (A) GFAP staining in coronal forebrain sections. Data are representative of 3 mice/genotype. Scale bar, 200 nm. (B) Quantification of the data shown in (A). Means ± S.E.M.; n=3. (C) Anatomical location of the panels shown in (A).

Figure 6. Increased density of microglia in brains of *Csflr*^{+/-} male mice

(A) Iba1 staining illustrating the density of microglia in different areas of the brain. Scale bar: 50 µm. (B) Quantification of the density of microglia in prefrontal and hippocampal sections. Grey bars, *Csflr*^{+/+}, green bars, *Csflr*^{+/-}. Means ± S.E.M.; n=3; *, p<0.05 compared to *Csflr*^{+/+} controls, Student's two-tailed t test. M1, primary motor cortex; M2, secondary motor cortex.

Figure 7. Increased frequency of CSF-1R⁺ neurons in aging mice

(A) Forebrain sections stained with antibodies against NeuN (green), CSF-1R (red) and counterstained with DAPI (blue). The arrows point to examples of CSF-1R⁺ NeuN⁺ cortical neurons. Scale bar: 50 μ m. (B) Quantification of the percentage of CSF-1R⁺ NeuN⁺ neurons in different layers of the cortex. Gray bars, *Csflr*^{+/+}, green bars, *Csflr*^{+/-}. Means $\pm$ S.E.M.; n=3 mice/group. Irrespective of the mouse genotype, *Csflr* expression increases with age in cortical layers V ($F_{1,8} = 86.85$, $p < 0.0001$) and VI ($F_{1,8} = 18.11$, $p = 0.0028$). The p-values for the difference between the *Csflr*^{+/-} and *Csflr*^{+/+} control groups or between age groups were determined using Tukey's post-hoc analysis. *, $p < 0.05$; **, $p < 0.01$.

Figure 8. Altered cytokine, chemokine and receptor expression profiles in *Csf1r*^{+/-} forebrains
 Except where otherwise indicated, estimations were performed on mRNA or protein from the anterior motor cortex and corpus callosum of 12 month-old mice. (A) Quantitative RT-PCR for the CSF-1R and its ligands (n=2). (B) Western blots for the CSF-1R, showing expression of mature (~165 kDa) and immature (~135 kDa) forms of the CSF-1R. (C) Quantitation of the CSF-1R western blot data (n=2). (D) Concentrations of CSF-1 and IL-34 in various brain regions of seven week-old mice determined by ELISA. Means $\pm$ range; n=2 mice/genotype. (E, F) *Csf1r* haploinsufficiency-associated changes in the expression of mRNAs of inflammatory cytokines, chemokines and receptors identified by qRT-PCR at 7 weeks (E) and 12 months (F) of age. Beta actin and GAPDH were used as house keeping gene standards in all experiments. Means $\pm$ S.E.M.; n=3 mice/genotype. The two-sided moderate t-test was performed using the LIMMA package in Bioconductor, while accounting for batch effects, where appropriate. *, p<0.05 vs *Csf1r*^{+/+}. HP, hippocampus; OBCx olfactory bulb and accessory cortex; aMCx, anterior motor cortex; aCC, anterior corpus callosum; pMCx, posterior motor cortex; pCC, posterior corpus callosum. Grey bars indicate *Csf1r*^{+/+}, green bars indicate *Csf1r*^{+/-}.

Figure 9. Model of cellular interactions contributing to neurodegeneration in aging *Csf1r*^{+/-} mice

The upper panel depicts the neuroprotective actions of the CSF-1R in aging wild type mice. Upregulation of CSF-1R on aging neurons (Fig. 7) is neuroprotective (Luo et al., 2013; Nandi et al., 2012). In microglia, CSF-1R signaling promotes a quiescent phenotype. These microglia may produce neuroprotective factors, keeping the balance between age-related neurodegeneration and survival in favor of survival. The lower panel illustrates effects of insufficient CSF1R expression in *Csf1r*^{+/-} mice. Insufficient CSF1R signaling in *Csf1r*^{+/-} neurons leads to more rapid neurodegeneration (Figs 3, 4C, 7). These neurons are hypermyelinated (Fig 4A, B) and upon their death, increase the autoantigenic load, leading to inflammation and possibly to autoimmunity. Stimulation of *Csf1r*^{+/-} microglia by neuronal debris in the presence of increased GM-CSF and decreased CSF-1R signaling induces an activated dendritic cell-like state with the production of neurotoxic factors, such as IL-6 (Fig 8F) (Fischer et al., 1993; Smith, 1993). Concomitant upregulation of IL-6 and IL6Rα in the brain (Fig 8F) enhances IL-6 trans-signaling ultimately leading to neurotoxicity (Campbell et al., 2014). This establishes a feedback loop that enhances neurodegeneration.