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BRAIN RESEARCH

Research Report

Axl–/– mice have delayed recovery and prolonged axonal damage following cuprizone toxicity

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ARTICLE INFO

Article history:
Accepted 18 August 2008
Available online 5 September 2008

Keywords:
Copper toxin
Oligodendrocyte maturation
Phagocytosis
Vesicle
Axonal damage
Myelination
Autophagy

ABSTRACT

Activation of the receptor tyrosine kinase Axl recruits signaling molecules that regulate cell growth and survival. To evaluate Axl's role in brain during cuprizone toxicity, mice were fed cuprizone and evaluated at 3-, 4-, and 6-week cuprizone treatment and 3- and 5-week post-cuprizone withdrawal. At 4-week cuprizone treatment, the corpora callosa of wildtype (WT) mice had robust Oil Red O+ staining indicative of ongoing phagocytosis. Axl–/– mice had minimal Oil Red O+ staining, fewer microglia, and significantly more TUNEL+/ASPA+ mature oligodendrocytes than the WT. At 6-week cuprizone treatment, there was significantly more Oil Red O+ staining in the Axl–/– corpora callosa than in the WT indicating a lag in the clearance of cellular and myelin debris. Relative to WT mice, there were fewer mature oligodendrocytes and significantly more SMI-32+ axons at 3-week post-cuprizone withdrawal, indicative of axonal damage in the Axl–/– corpora callosa. Electron microscopy determined that at 3-week post-cuprizone withdrawal the number of dystrophic axons and axons containing autophagosome-like vacuoles/mouse was increased in the Axl–/– mice relative to the WT mice. In Axl–/– corpora callosa, 5-week post-cuprizone withdrawal, the number of mature oligodendrocytes was comparable to the WT mice, but axons in the Axl–/– mice were SMI-32+, suggesting that Axl–/– mice have delayed clearance of apoptotic oligodendrocytes and myelin debris resulting in prolonged axonal damage and recovery from cuprizone toxicity.

1. Introduction

Axl, Tyro3, and Mer, comprise a family of cell adhesion molecule-related receptor tyrosine kinases (RTKs). The receptors are expressed on cells of the immune, nervous and reproductive systems where co-expression of one or more family member is often detected on the same cell type (Lai and Lemke, 1991; O'Bryan et al., 1991; Graham et al., 1994; Lai et al., 1994; Prieto et al., 2000; Lemke and Lu, 2003). During rat brain development and myelination, *in situ* hybridization determined that Axl was highly expressed in white and gray matter, in oligodendrocytes, neurons, Schwann cells, and endothelial

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Abbreviations: Gas6, growth arrest-specific protein 6; DAPI, 4′,6-diamidino-2-phenylindole; PI3 kinase, phosphatidylinositol 3 kinase; TNFα, tumor necrosis factor α; cuprizone, bis-cyclohexanone oxalydihydrazone; kDa, kilodalton; LFB, luxol fast blue; RTK, receptor tyrosine kinase; mAb, monoclonal antibody; pAb, polyclonal antibody; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; DAB, diaminobenzidine; EM, electron microscopy; WT, wildtype; OPCs, oligodendrocyte progenitor cells; MBP, myelin basic protein; APC, adenomatus polyposis coli; ASPA, aspartoacylase; APP, β-amyloid precursor protein; MA, macroautophagy

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doi:10.1016/j.brainres.2008.08.076

cells (Li et al., 1996; Avanzi et al., 1998; Prieto et al., 1999, 2000; Shankar et al., 2003; Prieto et al., 2007). Our lab confirmed the expression of Axl, Tyro3 and Mer on O4+ immunopanned oligodendrocytes by RT-PCR, and microarray analysis, and Axl by Western blot analysis (Shankar et al., 2003). Also, Axl is expressed on murine microglia (Weinger et al., 2008).

The ligand for Axl is growth arrest-specific protein 6 (Gas6) (Bellosta et al., 1997; Goruppi et al., 1999). Gas6 is widely expressed in the nervous system, and is secreted by neurons and endothelial cells (Stitt et al., 1995; Varnum et al., 1995; Crosier and Crosier, 1997; Avanzi et al., 1998; Allen et al., 1999; Prieto et al., 2000); Gas6 is concentrated along the plasma membrane in resting endothelial cells (EC) (Avanzi et al., 1998). The relative affinity of Gas6 for Axl, Tyro3, and Mer receptors is Axl>Tyro3>Mer. The receptor binding site of Gas6 maps to the two Ig-domains of Axl, and Gas6 is the sole ligand for Axl (Ebner et al., 2000). Although Gas6 can activate the receptors, receptor monomers on the surface of opposing cells have been shown to dimerize through homophilic interactions with a large cluster of molecules generating a cooperative effect sufficient for stable adhesion (Li et al., 1996; Bellosta et al., 1997; Goruppi et al., 1999; Heiring et al., 2004).

Several studies have demonstrated that signaling through the Axl receptor can protect various cell types from cell death. The anti-apoptotic effect of Axl signaling was confirmed using fibroblasts from Axl–/– mice showing that the absence of Axl resulted in higher levels of serum deprivation-induced apoptosis that could not be rescued by the addition of Gas6 (Bellosta et al., 1997). Our *in vitro* studies showed that Gas6 treatment of human oligodendrocytes enhanced maturation resulting in the expression of mature oligodendrocyte markers (Shankar et al., 2003), and enhanced survival by activating the PI3 kinase/AKT signaling pathway, thereby protecting against growth factor withdrawal and tumor necrosis factor α (TNFα)-cytotoxicity (Shankar et al., 2006).

In this study, we used the cuprizone model to test the hypothesis that when challenged with the copper chelator, cuprizone (bis-cyclohexanone oxalydihydrazone), recovery in the Axl–/– mice would be compromised relative to WT littermates. Cuprizone (0.2% w/w) administered in mouse chow induces mature oligodendrocyte cell death, and demyelination of the corpus callosum by 3 weeks following ingestion. In addition to the death of mature oligodendrocytes, axons shrink in size as a result of demyelination, micro- and macrogliosis occurs, and the expression of several cytokines and chemokines are upregulated, peaking at 4 weeks of cuprizone treatment (Blakemore 1973a,b; Hiremath et al., 1998; Mason et al., 2001; Matsushima and Morell, 2001; Jurevics et al., 2002; Sun et al., 2006). The withdrawal of cuprizone from the diet at 6 weeks results in the repopulation and maturation of oligodendrocytes from oligodendrocyte progenitor cells (OPCs). In WT mice, remyelination nears completion 3 weeks after cuprizone withdrawal (Blakemore, 1973a,b; Hiremath et al., 1998; Mason et al., 2001; Matsushima and Morell, 2001; Jurevics et al., 2002). We compared the Axl–/– and WT mice at 3-, 4- and 6 weeks of cuprizone treatment, and 3- and 5-week post-cuprizone withdrawal. During cuprizone toxicity we examined whether the deletion of Axl altered the number of mature oligodendrocytes or microglia, or impacted on phagocytosis of myelin and cellular debris. During the recovery phase we examined whether the deletion of Axl altered the maturation of oligodendrocytes, axonal diameter, myelin thickness or *g*-ratios, limited remyelination, or resulted in prolonged axonal damage within the corpus callosum relative to WT littermates.

2. Results

Prior to performing our cuprizone studies, we examined whether Axl and WT littermates have equivalent mature oligodendrocytes in the corpus callosum at 17 weeks of age. Sections from WT and Axl–/– mice were examined at the corpus callosum using the monoclonal antibody (mAb) CC1 generated against the mature oligodendrocyte marker APC. Double stained CC1+/DAPI+ oligodendrocytes were counted within the corpus callosum. No difference in the two groups of mice was observed prior to the initiation of cuprizone treatment. The number of CC1+/DAPI+ oligodendrocytes in the untreated WT littermate group ($N=2$) was 1321/mm^2, and the number of CC1+/DAPI+ oligodendrocytes in the Axl–/– group of mice ($N=2$) was 1272/mm^2.

Fig. 1 illustrates the cuprizone-induced demyelination in the corpus callosum of Axl–/– and WT littermates after 6 weeks on cuprizone, and 3 weeks following the removal of cuprizone from the diet. To monitor the extent of demyelination at 6 weeks and to determine whether myelination was complete 3 weeks following the removal of cuprizone, brain sections were incubated with the myelin basic protein (MBP) mAb SMI99. Robust myelin was observed in the untreated WT (A) and Axl–/– mice (B), and diminished myelin was apparent 6 weeks after cuprizone ingestion (C, D). Three weeks following the removal of cuprizone, remyelination was observed in the WT littermate controls (E) and qualitatively less remyelination was detected in Axl–/– mice (F).

To evaluate whether the Axl–/– group of mice quantitatively demonstrated a lag in remyelination, MBP and luxol fast blue (LFB) staining of sections from the WT and Axl null animals was performed 3 weeks following cuprizone withdrawal. Myelination was microscopically scored by examining multiple LFB-stained sections of the corpus callosum of each mouse and comparing the extent of remyelination to the powdered chow control mice. An arbitrary score of 0–4 was used to score remyelination, with a score of 4 equivalent to 100% remyelination, and a score of 0 indicative of no myelination. Our analysis included sections from 17 Axl–/– mice and 8 WT mice. Examination of sections determined that the mean value for the Axl–/– mice was 3.38+/−0.175, or approximately 84.5% remyelination ($N=17$). In 5/17 of the null mice remyelination ranged from 43.8%–75%; in the remaining 11/17 Axl–/– null mice remyelination was >75%. The mean overall score for the WT mice was 3.64+/−0.10, or ~91% remyelination ($N=8$). Seven of eight of the WT corpora callosa had near complete remyelination, the one exception had 75% remyelination. Based upon an unpaired two-way *t*-test for two-way ANOVA and Bonferroni test, there was no statistical difference between the two groups of mice ($p>0.05$). These data were consistent with the MBP staining of the same Axl–/– and WT mice which showed a non-statistically significant decrease in remyelination based on similar scoring. To