Published in final edited form as:

Glia. 2017 December ; 65(12): 2051–2069. doi:10.1002/glia.23214.

Loss of Gas6 and Axl signaling results in extensive axonal damage, motor deficits, prolonged neuroinflammation and less remyelination following cuprizone exposure

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Abstract

The TAM (Tyro3, Axl, and MerTK) family of receptor tyrosine kinases (RTKs) and their ligands, Gas6 and ProS1, are important for innate immune responses and central nervous system (CNS) homeostasis. While only Gas6 directly activates Axl, ProS1 activation of Tyro3/MerTK can indirectly activate Axl through receptor heterodimerization. Therefore, we generated Gas6^{-/-}Axl^{-/-} double knockout (DKO) mice to specifically examine the contribution of this signaling axis while retaining ProS1 signaling through Tyro3 and MerTK. We found that naïve young adult DKO and WT mice have comparable myelination and equal numbers of axons and oligodendrocytes in the corpus callosum. Using the cuprizone model of demyelination/remyelination, transmission electron microscopy revealed extensive axonal swellings containing autophagolysosomes and multivesicular bodies, and fewer myelinated axons in brains of DKO mice at 3-weeks recovery from a 6-week cuprizone diet. Analysis of immunofluorescent staining demonstrated more SMI32+ and APP+ axons and less myelin in the DKO mice. There were no significant differences in the number of GFAP+ astrocytes or Iba1+ microglia/macrophages between the groups of mice. However, at 6-weeks cuprizone and recovery, DKO mice had increased proinflammatory cytokine and altered suppressor of cytokine signaling (SOCS) mRNA expression supporting a role for Gas6-Axl signaling in proinflammatory cytokine suppression. Significant motor deficits in DKO mice relative to WT mice on cuprizone were also observed. These data suggest that Gas6-Axl signaling plays an important role in maintaining axonal integrity and regulating and reducing CNS inflammation that cannot be compensated for by ProS1/Tyro3/MerTK signaling.

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Keywords

neuroinflammation; axonal damage; motor deficits; TAM signaling; demyelination

Introduction

Multiple Sclerosis (MS) is a disease of unknown etiology in which the body's immune cells target myelin resulting in wide-ranging symptoms including fatigue, muscle weakness, numbness and paralysis in over 2.3 million people worldwide (Noseworthy et al., 2000; Sospedra et al., 2005). Axonal damage and demyelination are recognized as significant contributors to MS disease progression and disability (Bitsch et al., 2000; Ferguson et al., 1997; Garbern et al., 2002; Hemmer et al., 2002; Kornek et al., 2000). Major obstacles for individuals with MS are maintenance of axonal function, mobility and the ability to efficiently remyelinate axons following bouts of demyelination. Current MS treatments modulate immune responses but are ineffective at protecting against immune-mediated damage or aiding in CNS repair. Therefore, understanding the cell signaling pathways that regulate the survival of CNS-resident cells during and after an inflammatory attack is paramount for therapeutic development. While no perfect mouse model of MS exists, several models attempt to examine various aspects of the observed CNS pathology. The cuprizone model employed in our studies provides a reproducible manner to study demyelination, inflammation, axonal damage and myelin repair/remyelination (Hiremath et al., 1998; Mason et al., 2000; Schultz et al., 2017).

Tyro3, Axl, MerTK (TAMs) and their respective ligands, Gas6 and ProS1, play major roles in regulating inflammation and homeostasis (Lemke et al., 2008; Rothlin et al., 2007). Gas6 is the sole ligand for Axl; however, Axl can bind or heterodimerize with other receptors including Tyro3, MerTK, epidermal growth factor receptor (EGFR), and type I interferon receptor (IFNAR) to initiate intracellular signaling (Rothlin et al., 2007; Vouri et al., 2016). Gas6 is highly expressed in the healthy mouse CNS by microglia, endothelial cells and neurons (Prieto et al., 1999; Zhang et al., 2014). While Axl is expressed by astrocytes, oligodendrocyte progenitor cells (OPCs) and to a lesser degree on other resident CNS cells, it can be upregulated by microglia during certain pathological conditions and inflammation (Fourgeaud et al., 2016; Prieto et al., 2000; Yin et al., 2017; Zhang et al., 2014). Expression of ProS1 is seen in microglia and endothelial cells (Zhang et al., 2014) and along with MerTK and Gas6 are highly expressed in homeostatic microglia (Butovsky et al., 2014).

The importance of both Gas6 and Axl signaling has been well documented in the CNS during autoimmune and demyelinating conditions such as MS and in mice during myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) and the cuprizone toxicity model (Akkermann et al., 2017; Binder et al., 2008; Goudarzi et al., 2016; Gruber et al., 2014; Hoehn et al., 2008; Shankar et al., 2006; Tsperson et al., 2010; Weinger et al., 2011; Weinger et al., 2009). Our study of TAM proteins in MS lesions showed elevated levels of soluble Axl and MerTK, and increased levels of proteins associated with Axl and MerTK solubilization that is not seen in normal appearing white matter from the same individuals (Weinger et al., 2009). Soluble Axl and

MerTK are known to act as decoy receptors and block Gas6 binding to membrane-bound receptors. Dysregulation of protective Gas6 receptor signaling is thought to prolong lesion activity. To eliminate Gas6 signaling, including abolishing Axl activation via interaction with other receptors, we generated Gas6^{-/-}Axl^{-/-} double knockout (DKO) mice to specifically examine the contribution of this signaling axis, while retaining ProS1 signaling through Tyro3 and MerTK. While others and our laboratory have examined Axl^{-/-} KO and Gas6^{-/-} KO mice in the cuprizone model, the motor impairment, extensive axonal damage, pathology, and prolonged cytokine expression that we observe in the corpus callosum of DKO mice have not been reported (Binder et al., 2008; Hoehn et al., 2008). Thus, complete deletion of the Gas6 signaling pathway and inability of Axl to crosstalk with other receptors appears to significantly impact resolution of inflammation, axonal integrity and remyelination, which cannot be compensated for by ProS1/MerTK/Tyro3 signaling.

Materials and Methods

Generation of Gas6^{-/-}Axl^{-/-} DKO Mice

Gas6^{-/-} mice, obtained from Dr. Pablo deFrutos (Institute of Biomedical Research of Barcelona), were mated with Axl^{-/-} mice, obtained from Dr. Greg Lemke (Salk Institute for Biological Studies), to generate Gas6^{-/-}Axl^{-/-} mice. All mice were extensively backcrossed onto a C57Bl/6J background prior to mating. Multiple independently generated DKO mice were studied. Similar results were obtained from different parental lines. Wild type (WT) C57Bl/6J mice were used as controls. The Animal Care and Use Committee at Albert Einstein College of Medicine in compliance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals approved all animal protocols.

Genotypes were confirmed using duplexed PCR assays for both WT and knockout (KO) alleles (Platinum PCR SuperMix, ThermoFisher Scientific) for each gene followed by gel electrophoresis. The following PCR cycling parameters were used: 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute with a final extension step of 72°C for 5 minutes and 4°C hold. All primers were used at 10μM. Primer sequences are listed in Table 1.

Cuprizone treatment

Mice were fed 0.2% (w/w) cuprizone (Sigma; St. Louis, MO) in powdered chow *ad libitum* for 6 weeks. Food was changed on a Monday, Wednesday, Friday schedule followed by regular chow pellets for an additional 1, 3 or 6 weeks. Mice were sacrificed at 4-weeks, 5-weeks and 6-weeks cuprizone-supplemented diet, and during the recovery phase, 1-, 3-, or 6-weeks after the removal of cuprizone from the diet. For immunohistochemical analysis, brains were removed following cardiac perfusion with 4% paraformaldehyde, and paraffin-fixed and frozen sections were prepared. Coronal sections of brain (10μm) were cut on a cryostat and the midline of the corpus callosum was evaluated at the region of the fornix corresponding to sections 220–260 of Sidman's mouse atlas (Sidman et al., 1971).

Electron microscopy

Following cardiac perfusion with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M cacodylate buffer, the brain was removed, and the area immediately encompassing the midline of the corpus callosum was cut into 1mm sections and oriented such that cross sections of axons within the corpus callosum were obtained. Ultrathin sections were cut, stained with uranyl acetate and lead citrate. Photographs of the axons cut in cross section were obtained and minimally 5 fields of $\times 5000$ non-serial sections were used to calculate the mean number of myelinated axons/ $\times 5000$ sheet. Print magnification was $\times 13,500$. Image J software was used to calculate the axonal diameter, myelin thickness and g-ratios of 3–4 animals; 100 axons/mouse were counted. The g-ratio represents the ratio of the diameter of the axon to the diameter of the axon plus myelin.

Immunostaining

Sections were incubated with CC1, a monoclonal antibody (mAb) to the mature oligodendrocyte marker APC (adenomatous polyposis coli, clone CC1, 1:10; Chemicon/EMD Millipore; La Jolla, CA). Antibodies used to examine bHLH transcription factors Olig1 and Olig2 were obtained from Dr. John Alberta (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA). Oligodendrocyte progenitor cells (OPCs) were identified by the OPC marker PDGFR α supplied by Dr. William Stallcup (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA). Myelin basic protein (MBP) was detected in paraffin-embedded tissue using monoclonal antibody SMI94 (EMD Millipore) and the mean fluorescent intensity (MFI) was determined in Image J. Astrocytes were examined using GFAP polyclonal antibody (1:200; EMD Millipore). Iba1 polyclonal antibody (pAb) was used as a microglia/macrophage marker (1:400; WAKO, Richmond, VA). Amyloid Precursor Protein (APP) immunostaining (1:100, Y188, Abcam, USA) and non-phosphorylated neurofilament protein immunostaining using SMI-32 (1:20,000; EMD Millipore) were performed on frozen sections. All sections were incubated for 30 min with 1 $\times$ Tris-buffered saline pH 7.4 (1 $\times$ TBS) containing 0.25% Triton X-100, followed by a 1 h incubation in 5% goat serum and 5% nonfat dry milk in 1 $\times$ TBS, and incubated with antibodies diluted in 5% nonfat dry milk in 1 $\times$ TBS, overnight at 4°C. Stained sections were washed with 1 $\times$ TBS + 0.1% Tween and incubated with secondary antibody directly conjugated to Alexa-fluor (Invitrogen; Eugene, OR) for 1 h, washed 3 times in 1 $\times$ TBS, followed by 10 min incubation with 4',6-diamidino-2-phenylindole (DAPI; 1:1000; Sigma), followed by 3 washes with 1 $\times$ TBS, and mounted with Biomedica aqueous mounting medium (Biomedica, Inc.; Foster City, CA). Light microscope images were collected on an Olympus IX81 fluorescent microscope with a Sensicam QE cooled CCD camera using the 20x or 60x magnifications. All images were taken at the midline of the corpus callosum. The cell counts are presented as the number of cells/mm² based on the magnification at: 20x = 0.149 mm² and 60x = 0.0155 mm². Apoptosis was measured via TUNEL staining according to the manufacturer's instructions (Click-iT Plus TUNEL Assay; Invitrogen; Carlsbad, CA).

Oil Red O staining

Frozen sections were incubated in distilled water for 1 min followed by a 2-min incubation in 100% propylene glycol (Polyscientific; Bayshore, New York) whereupon the sections

were transferred to Oil Red O stain for 48 hours at room temperature. Sections were rinsed in double-distilled deionized (dd)H₂O for 2 minutes, incubated for 1 min in 85% propylene glycol and mounted with gelatin mounting medium. Mounted cross sections were scanned on a Perkin Elmer P250 Panoramic Slide Scanner. Slides were imaged with a 4-megapixel-color CMOS camera with a 20x objective and then stitched together. Images were analyzed using Panoramic Viewer software.

Myelin Black Gold II Staining

Myelin staining of frozen sections was performed using the Myelin Black Gold-II Staining Kit (EMD Millipore) according to the manufacturer's instructions. Sections were incubated in ddH₂O for 2 minutes then incubated in a moist chamber at 60°C for 12 minutes in pre-warmed Black Gold-II solution. The slides were rinsed twice in ddH₂O, incubated in pre-warmed 1% sodium thiosulfate for 3 minutes, followed by three washes in ddH₂O. After staining, the slides were mounted with aqua-mount. Stained and mounted sections were scanned, visualized and analyzed in the same manner as the Oil Red O stained slides.

Quantitative Real-Time PCR

Total RNA was extracted from freshly isolated corpora callosa using the TRIzol reagent (Ambion; Waltham, MA) following the manufacturer's instructions. RNA concentration and purity was determined using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher) at an absorbance ratio of 260 and 280 nm. cDNA was synthesized using 1 µg of RNA reverse transcribed using iScript cDNA synthesis kit (Bio-Rad; Hercules, CA). For qRT-PCR reactions, templates were diluted 1:15 in a PCR mix containing each gene-specific primer pair and iTaQ Universal SYBR Green Supermix (Bio-Rad). To ensure accuracy of cDNA amplification, PCR primers were generated to span an intron. Gene expression was measured on a Step One Plus Real-Time PCR machine (Thermo Fisher). Each sample was run in triplicate and values obtained were normalized to the levels of hypoxanthine-guanine phosphoribosyltransferase (HPRT). Normalization was validated in select samples using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold induction was calculated as $2^{-\Delta\Delta CT}$. Oligonucleotide primers used for qRT-PCR are listed in Table 1.

Behavioral Testing

Negative Geotaxis: The negative geotaxis assay assesses proprioceptive and righting reflexes by measuring latency to right (in seconds) when placed facing nose downward to nose upward on a grid screen placed at a 90° angle or perpendicular to the floor (Rogers et al., 1997). Negative geotaxis was performed on untreated mice of both genotypes (naïve - prior to cuprizone ingestion) and at 5-weeks and 6-weeks while receiving cuprizone. ***Balance beam assay:*** A set of balance beams was used to test motor coordination by assessing the number of slips made while crossing round wooden beams of varying diameters (1.3-cm 1.8-cm and 2.3-cm) in which larger diameter beams are easier. ***Object Placement:*** The object placement test of spatial working memory (Ennaceur et al., 1988; Ennaceur et al., 1992) is based on the natural and robust tendency of rodents to preferentially explore novel objects (Ennaceur et al., 1988). The assay was performed essentially as described previously (Ennaceur et al., 1988; Ennaceur et al., 1992). Briefly, in Trial 1, mice were placed in an opaque plastic arena (16" square) and allowed to freely explore 2 identical, non-toxic

objects (such as plastic, glass or ceramic items) for 6 minutes. High contrast visual cues were placed on the walls of the open field. The time spent exploring the objects was recorded manually with timers, after which the animal was returned to the home cage. Following a retention interval of 25 min, the animal was returned to the arena in which one of the objects was displaced in space (Trial 2). Care was taken to ensure that the intrinsic relationship between the objects was changed, and not just the position relative to a visual cue. The mouse was again allowed to explore for 3 min, during which time the exploration of both the displaced (novel) and familiar objects was scored. Preference scores are illustrated as the percent of exploration of the new object/exploration of new+old, thus 50% exploration indicates no preference for the novel object. Results are also shown as the proportion of subjects passing and failing where passing criteria is a 55% preference score. This rationale and validation of this criterion has been detailed elsewhere (Cole et al., 2013; Li et al., 2010a; Li et al., 2010b). There was no significant difference in motor performance of naïve WT and DKO mice on the balance beams, negative geotaxis, and object placement assays. Testers were blinded to the genotype of the mice.

Statistical Analysis

All values are presented as mean±standard error of the mean (SEM). Statistical analyses were performed in GraphPad Prism Software. Significance was determined using a two-tailed unpaired student's t-test for parametric analyses and Mann-Whitney for nonparametric analyses with $p < 0.05$ as the threshold of significance. For behavioral testing, values obtained in each test were statistically analyzed by Mann-Whitney test or Chi-squared analysis in GraphPad Prism Software.

Results

Naïve WT and DKO mice exhibit similar myelination and CNS architecture

Gas6^{-/-} single knockout mice were mated with Axl^{-/-} single knockout mice to generate the Gas6^{-/-}Axl^{-/-} double knockout (DKO) mouse strain. Establishment of the DKO mice was confirmed by PCR analysis (Figure 1A). To determine whether deletion of both Gas6 and Axl cause any overt phenotypic differences in CNS architecture and myelination in naïve mice, brain and spinal cord sections were examined by histology, immunostaining, and/or electron microscopy at multiple developmental time points. At 10-weeks of age, there were no differences in brain architecture of WT and DKO mice by hematoxylin and eosin (H&E) staining (Figure 1B), nor were there any differences in myelination by MBP immunostaining of the corpus callosum (arrow) and lumbar spinal cord. Equivalent numbers of cytoplasmic Olig1+ oligodendrocytes (OLs; ~1064 cells/mm²) at the midline of the corpus callosum of naïve WT and DKO mice by immunofluorescent (IF) staining and light microscopy were observed (Figure 1C), indicative of mature OLs (H. A. Arnett et al., 2004). Additionally, by immunostaining for APC+/CC1+ mature OLs, we found 1266.5±113.6 CC1+ cells/mm² and 1223.6±64.4 CC1+ cells/mm² in naïve DKO and WT corpora callosa, respectively (Figure 6A). By transmission electron microscopy (TEM), the number of myelinated axons at the midline of the corpus callosum (x5000 contact sheets) was equivalent in DKO (219.3±24.9 axons) and WT (228.9±12.4 axons) mice (Figure 1D). Additional measurements presented in Table 2 as micrometers±SEM, determined that myelin thickness was not significantly

different between the naïve DKO (0.203 ± 0.013) and WT (0.250 ± 0.044) corpora callosa, $p=0.36$; nor was the axonal diameter in DKO (0.700 ± 0.051) and WT (0.729 ± 0.088) mice, $p=0.79$. The g-ratio of the DKO (0.764 ± 0.017) and WT (0.739 ± 0.017) mice ($n=3$) were also not significantly different. Necropsy reports determined that naïve DKO mice have normal brain, spine, and spinal cord and all organs appeared in good health relative to age and sex-matched WT mice.

Less remyelination and extensive axonal damage is observed in DKO mice after 3-weeks recovery from cuprizone diet

To determine whether DKO mice are able to efficiently recover from cuprizone toxicity, we examined tissue pathology in the corpora callosa after 3-weeks recovery from cuprizone. Remyelination was evaluated in paraffin-embedded sections by staining for MBP and determining the mean fluorescent intensity (MFI; Figure 2Aa–b,B). MBP MFI was reduced in the corpora callosa of DKO mice (4302 ± 168.4) relative to WT mice (4977 ± 192.2 , $p = 0.05$; Figure 2B). This was accompanied by fewer mature cytoplasmic Olig1+ cells in the DKO (591.3 ± 45.0 cells/mm²; $n=11$) mice relative to WT (739.7 ± 48.4 ; $n=9$) mice ($p=0.027$; Figure 2Ac–d,C), as well as more cells staining for nuclear Olig1 in DKO (202.4 ± 26.02 cells/mm²; $n=7$) vs. WT (86.0 ± 27.2 cells/mm²; $n=6$; $p=0.033$) corpora callosa indicating ongoing remyelination in the DKO mice (Figure 2Ac–d,C).

To examine how deletion of Gas6 and Axl affected axonal integrity, we determined the number of SMI32+ axonal swellings ($> 3\mu\text{m}$) in the corpus callosum at 3-weeks recovery from cuprizone. WT mice had 138 swellings/mm² ± 32.7 ($n=7$) while DKO mice had significantly more swellings with $322.4/\text{mm}^2 \pm 74.5$ ($n=6$; $p=0.035$), indicative of prolonged axonal damage when Gas6-Axl signaling is absent (Figure 2Ae–f,D). SMI32+ swellings in the DKO corpora callosa were found to be significantly greater relative to WT mice in two independent studies with different groups of mice. These findings were corroborated by APP immunostaining, which showed that DKO mice have significantly more axonal swellings (300.5 swellings/mm² ± 31.8 , $n=6$) relative to WT (156.4 swellings/mm² ± 39.4 , $n=7$; $p=0.018$, Figure 2Ag–h,E).

The extent of remyelination and axonal damage were further confirmed by transmission electron microscopy (TEM), which showed fewer myelinated axons at the midline of the DKO corpus callosum (Figure 3Ae–f) relative to WT mice (Figure 3Aa–d). Both female and male DKO mice had significantly fewer myelinated axons than WT mice (Figure 3B,D), consistent with the observed reduction in MBP MFI seen by light microscopy. In agreement with the increased SMI32+ and APP+ staining in DKO mice (Figure 2), significantly more swollen axons and pathology were observed in DKO corpora callosa (Figure 3Ae–g and 3C,E) relative to WT. Although WT mice have some abnormal appearing axons in the corpus callosum (Figure 3Ab boxed and magnified in 3c–d), the size of the axons and the extent of the pathology were significantly less when compared with the swollen (white arrows 3Ae) and damaged axons in the DKO mice (compare Ac,d to Ag). Noteworthy are the extensive numbers of swollen, degenerating axonal spheroids filled with multivesicular bodies (MVBs) (black arrow in Figure 3Af,g) as well as autophagosomes (black asterisk) and autophagolysosomes (white asterisk). These abnormal, naked axons were not counted in

the g-ratio calculations since we do not include unmyelinated or naked axons in our calculations. Analysis of g-ratio, axonal diameter, and myelin thickness for the naïve mice and at 3-weeks recovery were performed (Table 2). The data showed no significant difference in the two groups of mice at 3-weeks recovery; however, there was a trend toward increased axonal diameter in the DKO mice (0.820 ± 0.060) at 6-weeks cuprizone+3-weeks recovery relative to the WT mice (0.735 ± 0.056), $p > 0.05$.

Zebra bodies and numerous lipid droplets are found at the midline of the corpus callosum of DKO mice at 6-weeks cuprizone+3-weeks recovery

Zebra bodies comprising amorphous electron dense inclusions containing clearer crystalline stacks of membranes believed to be composed of aggregated proteins, membranes and lipids (Hirano, 1971, 1997) were observed in the corpus callosum of WT and DKO mice (Figure 4a–b, black arrowheads). Differences were noted in their overall size, number, and location in the cell. Only one zebra body was observed in three of seven WT mice extensively examined by TEM (size range 6.1 to 24.9 microns). The zebra bodies appeared to be in cell processes not visibly connected to the cell bodies, but most processes likely arose from microglia/macrophages involved in phagocytosis of debris. Multiple zebra bodies (mean value 2.5 ± 0.087 bodies/X5000 contact sheet) were observed in four of seven DKO mice and tended to be larger in size than those seen in WT mice (size range 4.7 to 161.7 microns in diameter). Similar to the WT, zebra bodies were observed in cell processes and also in the cytosol of cells with a nucleus and morphologies consistent with microglia (Figure 4c) and oligodendrocytes (Figure 4f). A representative swollen, dystrophic axon (A, upper right of Figure 4c) with filamentous bundles, swollen and degenerating mitochondria, and numerous autophagosomes and autophagolysosomes was observed in the DKO mice. Associated with the surface of the dystrophic, swollen axon is a microglia/macrophage (M) with a typical elongated nucleus, a centriole and a large zebra body in the cytosol. The body of the microglia/macrophage is in direct contact with the axons (white box) as shown at higher magnification in Figure 4d. Also observed is a recognizable healthy oligodendrocyte with a visible nucleus and rich cytoplasm containing membrane specializations with an oligodendroglial tongue adjacent to thinly myelinated axons (black box, Figure 4e) indicative of remyelination (Raine, 1999). While the predominant cell type containing zebra bodies was consistent with microglia/macrophages in the vicinity of damaged axons and cells, we observed in the DKO corpora callosa some oligodendrocytes that contained zebra bodies; note the zebra bodies adjacent to the nucleus in the corpus callosum of a DKO mouse in Figure 4f. The presence of zebra bodies in oligodendrocytes suggests that cellular debris remained within damaged cells at a time point when the vast majority of WT oligodendrocytes have recovered and were producing new myelin.

Deletion of the Gas6-Axl signaling axis results in defective debris clearance following cuprizone diet

Efficient clearance of debris is critical for remyelination, repair, and resolving inflammation (Clarner et al., 2012). Therefore, the corpora callosa of DKO and WT mice were examined using Oil Red O stain to determine whether deletion of the Gas6-Axl signaling axis would affect clearance of neutral lipids during cuprizone diet and recovery. At 5-weeks cuprizone diet, moderate Oil Red O+ staining intensity is observed in both WT and DKO mice (Figure

5A); however, there were no noticeable differences. Staining intensity peaked at 6-weeks cuprizone diet with noticeable decline in lipid staining at 1-week recovery, and no visible Oil Red O+ droplets observed in the WT mice at 3-weeks recovery. By contrast, DKO mice displayed more lipid droplets relative to WT mice at both 1-week and 3-weeks recovery indicating that deletion of both *Gas6* and *Axl* signaling *in vivo* impairs debris clearance during recovery from cuprizone toxicity.

In addition to differences in lipid debris clearance seen by Oil Red O staining, we observed increased numbers of pathologic lipid droplets in the corpora callosa of DKO mice relative to WT mice at 6-weeks cuprizone+3-weeks recovery by TEM (Figure 5B, white arrowheads). As part of our analyses, we examined all the contact sheets in both groups of mice (~5–7 contact sheets/mouse) for the presence of at least one lipid droplet and determined the percentage of contact sheets containing lipid droplets/mouse and the mean number of lipid droplets/mouse. All DKO mice examined had lipid droplets. The percentage of lipid droplet positive contact sheets in DKO mice ranged from 50–100% and resulted in a mean value of $86.0\% \pm 9.8$ for all mice examined. This was higher than what was observed in the WT mice, which had a mean value of $55.8\% \pm 9.4$. Analysis of the mean number of lipid droplets/mouse showed DKO corpora callosa containing significantly more lipid droplets (4.3 ± 0.20) relative to WT mice (0.92 ± 0.08 ; $p=0.0001$).

Over time, deletion of the *Gas6-Axl* signaling axis does not affect the number of immature and mature CC1+ oligodendrocyte populations but does alter remyelination and corpus callosum thickness during recovery

Given the observed differences in myelination after 3-weeks recovery, we examined if there were differences in immature and mature OL numbers during cuprizone diet and recovery. Examination of the number of OPCs present at the midline at 5- and 6-weeks cuprizone diet and after 1- and 3-weeks recovery was performed using a polyclonal antibody to PDGFR α . At 5-weeks, both DKO and WT mice showed no significant difference in the number of PDGFR α + OPCs ($1677.98 \text{ cells/mm}^2 \pm 133.25$ vs. $1212.60 \text{ cells/mm}^2 \pm 239.87$, $p>0.05$; Figure 6A). While the number of PDGFR α + OPCs declined at 6-weeks ($963.34 \pm 39.15 \text{ cells/mm}^2$ in the DKO vs. $718.93 \text{ cells/mm}^2 \pm 155.97$ in the WT), 6-weeks + 1-week recovery ($553.84 \text{ cells/mm}^2 \pm 75.1$ in DKO vs. $515.2 \text{ cells/mm}^2 \pm 37.2$ in WT) and 6-weeks + 3-weeks recovery ($237.8 \text{ cells/mm}^2 \pm 66.4$ for DKO vs. $236.1 \text{ cells/mm}^2 \pm 42.9$ for WT), there were no observed significant differences. There was also no notable difference in the number of Olig2+ oligodendrocytes at the midline at 5-weeks cuprizone between DKO ($408.6 \text{ cells/mm}^2 \pm 43.0$) and WT mice ($451.6 \text{ cells/mm}^2 \pm 74.51$; $p=0.64$). The number of Olig2+ OLs increased at 6-weeks cuprizone in both DKO and WT ($632.9 \pm 143.3 \text{ cells/mm}^2$ vs. $723.3 \text{ cells/mm}^2 \pm 156.8$; $p=0.69$) and continued to increase after 6-weeks cuprizone + 1-week recovery ($1256.0 \text{ cells/mm}^2 \pm 114.9$ vs. $1194.0 \text{ cells/mm}^2 \pm 135.6$ for WT; $p=0.7$) and 3-weeks recovery ($1095.0 \text{ cells/mm}^2 \pm 78.4$ vs. $1063.0 \text{ cells/mm}^2 \pm 49.2$, $p=0.7$). Thus, at each time point examined, the number of immature PDGFR α + OPCs and total Olig2+ OLs were not significantly altered at the midline in DKO and WT corpora callosa. Analysis of CC1+ oligodendrocytes over the entire course of cuprizone diet and recovery found no statistical differences observed at any time point. Minimal CC1+ OLs were observed at 5-weeks cuprizone diet in both WT ($370.3 \text{ cells/mm}^2 \pm 154.1$) and DKO ($32.2 \text{ cells/mm}^2 \pm 32.2$) mice,

$p > 0.05$. Oligodendrocyte numbers increased at 6-weeks cuprizone diet with no significant differences observed between the WT ($686.9 \text{ cells/mm}^2 \pm 150.3$) and DKO mice ($563.5 \text{ cells/mm}^2 \pm 88.7$), $p > 0.05$. CC1+ OL populations reached levels comparable to their naïve counterparts after 1-week recovery (WT $1143.1 \text{ cells/mm}^2 \pm 106.4$ vs. DKO $966.0 \text{ cells/mm}^2 \pm 102.6$) and remained at these levels after 3-weeks (WT $1054.6 \text{ cells/mm}^2 \pm 72.9$ vs. DKO $982.1 \text{ cells/mm}^2 \pm 73.3$) and 6-weeks recovery (WT $1416.8 \text{ cells/mm}^2 \pm 111.5$ vs. DKO $989.6 \text{ cells/mm}^2 \pm 114.9$). No differences were seen in apoptosis measured by detecting the number of TUNEL+ cells during these time points (data not shown).

To better understand the remyelination kinetics, we stained frozen sections of the corpus callosum with Myelin Black Gold-II, an aurohalophosphate complex that stains myelin. WT and DKO mice showed minimal myelin staining at 5-weeks cuprizone with only myelin punctae visible at the midline (Figure 6B). At 6-weeks cuprizone, moderate myelin staining was observed in both groups of mice with no notable differences. Despite no differences in remyelination while on cuprizone, DKO mice displayed a noticeable lag in remyelination after 1- and 3-weeks recovery relative to WT. The decreased myelin staining observed in DKO after 3-weeks recovery is consistent with less MBP staining, fewer cytoplasmic Olig1+ oligodendrocytes (Figure 2), and fewer myelinated axons observed via TEM (Figure 3). Remyelination at 6-weeks recovery was performed to determine if DKO mice are able to sufficiently repair and remyelinate to a similar extent as WT mice over an extended recovery period. Staining at the midline of the corpus callosum showed robust remyelination after 6-weeks withdrawal in both DKO and WT mice (Figure 6C). However, despite the similar extent of remyelination, a pronounced and significant narrowing of the corpus callosum at the midline of DKO mice was observed ($120.10 \mu\text{m} \pm 4.75 \mu\text{m}$; $n=7$) relative to WT mice ($145.2 \mu\text{m} \pm 5.87$; $n=9$), $p=0.0066$. This narrower width in the DKO corpus callosum was not observed at earlier time points.

The number of astrocytes and microglia/macrophages at the midline of the corpus callosum are unaffected in DKO and WT corpus callosum

Astrocytes and microglia/macrophages are the predominant phagocytic and inflammatory cell populations in the CNS and can aid in remyelination (Olah et al., 2012; Parenti et al., 2010). Given the observed differences in debris clearance and axonal damage following recovery, we examined whether this may be partially due to differences in astrocyte and microglia/macrophage recruitment. Cells with a defined DAPI+ nucleus and GFAP+ cytosolic staining were counted as astrocytes and the number of GFAP+ astrocytes/ mm^2 at the midline of the corpus callosum is illustrated in Figure 7. Peak astrocyte numbers were observed at 5-weeks cuprizone with a gradual decrease in number over the remaining course of cuprizone diet and recovery through 6-weeks. The data show no significant difference in the number of GFAP+ astrocytes at any time point. Microglia/macrophage populations were determined by counting the number of DAPI+Iba1+ cells/ mm^2 at the midline. At 5-weeks cuprizone, peak Iba1+ immunopositive glia was observed with no significant differences in cell number between DKO and WT mice (1695.0 ± 618.1 vs. 2662.0 ± 337.4 , $p=0.24$; Figure 7). The number of Iba1+ glia began to decline at 6 weeks, with similar numbers in DKO and WT mice (1433.48 ± 298.66 vs. 1332.93 ± 570.45 , $p=0.76$). At 3 weeks post-recovery from cuprizone, fewer Iba1+ cells were observed at the midline in both DKO and WT mice

(369.4±50.5 vs. 354.6±102.4, $p=0.89$). These data indicate that deletion of both Gas6 and Axl did not affect recruitment of these glial populations to the site of demyelination.

DKO mice have increased pro-inflammatory cytokine expression at 6-weeks cuprizone treatment that is maintained following 1-week recovery from cuprizone

Despite no observable differences in astrocyte and microglia/macrophage populations, we wanted to examine whether there were any differences in proinflammatory cytokine expression during cuprizone diet and recovery which may at least partially account for the differences in remyelination and increased axonal damage. The mRNA expression *TNF- α* , *IFN- γ* , *IL-1 β* , and *IL-6* in the corpora callosa of WT and DKO mice were determined by qRT-PCR and normalized to HPRT. We first analyzed the expression of these cytokines in naïve animals, and found no significant difference in expression between naïve DKO and WT mice (Figure 8). Relative to naïve, *TNF- α* was elevated in both the DKO and WT corpus callosum at 4-, 5- and 6-weeks cuprizone diet and persisted after 1-week recovery (Figure 8A). In WT mice, *TNF- α* expression peaked at 5-weeks cuprizone, followed by a steady decrease at 6-weeks and during the 1-week recovery period. In the DKO, *TNF- α* expression was significantly higher than WT at 6-weeks cuprizone and was downregulated after 1-week recovery from cuprizone, but did not decrease to WT levels.

IFN- γ is known to have a deleterious effect on the remyelination process (Lin et al., 2006; Maña et al., 2006), and similar to *TNF- α* , *IFN- γ* mRNA in WT and DKO mice steadily increased between 4–5 weeks relative to their naïve counterparts. By 6-weeks cuprizone treatment, WT *IFN- γ* was downregulated to levels below that of 4-weeks, whereas DKO *IFN- γ* expression remained significantly elevated and remained so at 1-week recovery (Figure 8B). Over the same time course, *IL-1 β* expression increased in both groups of animals with peak expression at 5-weeks cuprizone treatment. At both 6-weeks cuprizone diet and 1-week recovery, DKO *IL-1 β* expression remained higher than WT, but significance was not achieved (Figure 8C).

In response to injury and to protect cells of the CNS from damage, IL-1 β , IFN- γ and TNF- α can induce neurons, astrocytes and microglia to produce IL-6 (Ringheim et al., 1995; Woodroffe et al., 1991). *IL-6* mRNA expression in DKO mice was significantly higher than WT at 4- and 6-weeks cuprizone, and remained elevated after 1-week recovery (Figure 8D). This increased *IL-6* expression suggests ongoing pathology in the DKO corpora callosa while WT mice recover.

TGF- β 1 is a differentiation factor for microglia, and protects neurons against injury and neurodegeneration (Brionne et al., 2003; Flanders et al., 1998; Henrich-Noack et al., 1996; Wiessner et al., 1993). TGF- β 1^{-/-} mice have fewer microglia in the CNS, develop late onset motor dysfunction, and have reduced Axl expression and increased neuronal cell death in the brain (Brionne et al., 2003; Butovsky et al., 2014; Flanders et al., 1998; Henrich-Noack et al., 1996; Wiessner et al., 1993). In light of the extensive axonal damage and pathology observed in the corpus callosum, we examined *TGF- β 1* mRNA expression throughout cuprizone diet and 1-week recovery in DKO and WT mice and observed no significant differences in expression (Figure 8E).

Suppressor of cytokine signaling (SOCS), specifically SOCS1 and SOCS3 are expressed primarily in immune cells and CNS resident cells and impact cytokine/chemokine production, microglia/macrophage and astrocyte activation, and immune cell infiltration (reviewed in (Baker et al., 2009)). In the periphery, Axl signaling dampens inflammation by inducing downstream signaling of SOCS1 and SOCS3 via IFNAR activation (Lemke et al., 2008). Therefore, we determined whether DKO mice could effectively regulate *SOCS1* and *SOCS3* mRNA expression. Compared to their naïve counterparts, *SOCS1* expression in WT corpora callosa was upregulated at 4-weeks and peaked at 5-weeks cuprizone (Figure 8F,G). At the 6-week time point and after 1-week recovery, *SOCS1* expression returned to baseline and was similar to naïve expression. By contrast, DKO mice failed to upregulate *SOCS1* at all measured time points. In the WT corpora callosa, *SOCS3* mRNA was significantly elevated at 4- and 5-weeks cuprizone diet relative to naïve WT mice and expression returned to baseline by 6-weeks cuprizone and following 1-week recovery. In the DKO corpus callosum, *SOCS3* mRNA expression was equivalent to WT expression levels at 4- and 5-weeks cuprizone, but was significantly higher than WT mice by 6-weeks cuprizone treatment and following 1-week recovery. The increase in *SOCS3* mRNA retained in the DKO corpus callosum is consistent with *TNF- α* , *IFN- γ* and *IL-6* expression. Taken together, the data suggest that DKO mice have elevated proinflammatory cytokine expression relative to WT that likely delays recovery and contributes to the observed axonal damage and delay in remyelination.

Given the role of TAM receptors and their respective ligands in modulating inflammatory responses, we examined their expression levels over the course of cuprizone diet and 1-week recovery. In WT mice, *Gas6* mRNA expression was highest at 4-weeks cuprizone treatment by ~2-fold relative to baseline (Figure 8H). Expression levels decreased at 5-weeks and continued to decrease through 1-week recovery. A similar expression profile was seen with *Axl* mRNA (Figure 8I). *Tyro3* expression profiles were similar in naïve WT and DKO mice (Figure 8J). However, WT mice showed a highly significant increase in *Tyro3* expression after 4-weeks cuprizone (~48.5-fold over baseline expression), which declined by 5-weeks and remained at naïve levels until 1-week recovery when it increased relative to naïve WT and DKO expression levels. At all time points examined, DKO corpora callosa showed minimal and non-significant increases in *Tyro3* expression relative to naïve DKO mice. *MerTK* expression was also assessed (Figure 8K). At 4-weeks cuprizone, WT mice showed a ~15-fold increase in *MerTK* mRNA which steadily decreased over the course of cuprizone diet, eventually returning to near-baseline levels after 1-week recovery. Relative to naïve DKO mice, *MerTK* expression was upregulated ~4-fold at 4-weeks cuprizone treatment and decreased expression over the remaining time course. Despite showing an upregulation of *MerTK* mRNA expression, the increases in expression were far lower than WT mice and returned to baseline at an earlier time point. Overall, the expression profiles of *Tyro3* and *MerTK* mRNA in WT and DKO are substantially different. The lack of *Gas6* and *Axl* as well as the failure to upregulate *Tyro3* and *MerTK* may contribute to the inability of DKO mice to effectively regulate inflammation during cuprizone diet and recovery.

Additionally, we addressed whether *Pros1* mRNA expression might compensate for the loss of *Gas6* and *Axl*, and found that *Pros1* mRNA in DKO corpus callosum was significantly higher at 4-, 5-, and 6-weeks of cuprizone relative to WT (Figure 8L). Analysis of the extent

of axonal damage and increased inflammatory cytokine expression observed in the DKO mice suggest that ProS1 signaling via Tyro3 and MerTK is not able to efficiently compensate for the loss of Gas6 and Axl. However, it may be sufficient for recruitment of macrophages/microglia to sites of injury as reflected in similar numbers of Iba1+ cells.

Deletion of Gas6 and Axl signaling cause motor and cognitive deficits in DKO mice during cuprizone-mediated demyelination

To determine whether deletion of Gas6-Axl signaling would affect sensorimotor performance during cuprizone toxicity, two behavioral tests were utilized: negative geotaxis and balance beam. The negative geotaxis test assesses the righting reflex by measuring the amount of time it takes for mice to rotate head upwards when placed head downwards on a vertical grid. At 5-weeks cuprizone (Figure 9A), DKO mice had an increased latency in the righting reflex (13.12 ± 8.49 seconds, $n=5$) relative to WT mice (3.86 ± 0.85 seconds, $n=6$; $p=0.54$, Figure 9A). After 6-weeks cuprizone, DKO mice had a significantly longer latency for the righting reflex (15.98 ± 5.44 seconds, $n=9$) compared to WT mice (2.16 ± 0.39 seconds, $n=6$; $p=0.03$).

Additional testing on three balance beams of different diameters identified differences in motor function between the DKO and WT mice (Figure 9B). The number of slips observed as mice crossed the beam was recorded. At 5-weeks cuprizone, which is the point of peak demyelination in the cuprizone model, both DKO and WT mice showed similar motor deficits on the narrower 1.3cm beam (15.75 ± 1.40 slips vs. 12.20 ± 2.30 slips, $p=0.27$). Testing on the wider 1.8cm and 2.3cm beams, which provided a larger surface area for movement, showed that DKO mice had significantly more slips on both beams compared to WT mice. On the 1.8cm beam, DKO mice had 7.92 ± 1.67 slips while WT mice had 2.30 ± 0.78 slips ($p=0.004$). On the 2.3cm beam, DKO mice had more slips (2.50 ± 0.98 slips) compared to WT mice (0.42 ± 0.15 slips, $p=0.02$). At 6-weeks cuprizone, which is when mice have begun to remyelinate, fewer overall slips were observed in both groups of mice. However, DKO had significantly worse motor impairment on the 1.3cm beam relative to WT (8.50 ± 0.40 slips vs. 2.00 ± 0.86 slips, $p=0.04$). DKO had more slips on the 1.8cm beam as well; however, statistical significance was not reached (2.88 ± 1.130 vs. 0.50 ± 0.35 , $p=0.1$). Both groups of mice showed similar motor function on the 2.3cm beam (0.33 ± 0.24 vs. 0.33 ± 0.21 , $p=0.99$). The differences in motor deficits in DKO mice at 6-weeks cuprizone diet was significantly different relative to the WT mice, but no differences were observed between the two groups of mice at 3-weeks withdrawal.

Cognitive function was assessed using the object placement test of visuospatial memory (Figure 9C). At 6-weeks cuprizone, DKO mice had a significantly lower preference for novel objects relative to WT mice (left panel; $p=0.003$). The number of mice having a preference for the novel object was significantly lower in DKO mice as well (right panel; $X^2=6.4$; $p<0.01$).

Discussion

To better understand how the Gas6-Axl signaling axis affects axonal integrity during recovery from demyelination and inflammation, we studied our newly generated DKO

alongside WT mice during acute cuprizone demyelination and remyelination. Following 3-weeks recovery from cuprizone, DKO mice had significantly more axonal damage, fewer myelinated axons and significantly less myelin relative to WT mice. Additionally, DKO mice had decreased numbers of cytoplasmic Olig1+ OLs and higher numbers of nuclear Olig1+ OLs indicative of ongoing remyelination. This was further confirmed by the presence of OL processes in contact with unmyelinated axons in the DKO corpus callosum by TEM. Similar to the *Axl*^{-/-} and *Gas6*^{-/-} single KO mice, there was no significant difference in axon diameter, myelin thickness and g-ratio of DKO mice compared to WT (Binder et al., 2008; Hoehn et al., 2008). While *Axl*^{-/-} mice show no differences in the number of myelinated axons in the corpus callosum at 3-weeks recovery, significantly fewer myelinated axons are observed in DKO relative to WT mice, suggesting that deletion of *Axl* alone does not directly impair remyelination (Hoehn et al., 2008). The ability of *Axl*^{-/-} mice to efficiently remyelinate axons at 3-weeks recovery while *Gas6*^{-/-} and DKO mice show impairment in remyelination provide further support of a role for *Gas6*-*Tyro3* signaling in remyelination (Akkermann et al., 2017; Goudarzi et al., 2016; Prieto et al., 2007; Prieto et al., 2000).

Axonal damage was not reported in cuprizone-treated *Gas6*^{-/-} mice (Binder et al., 2008; Binder et al., 2011); however, we observed swollen axons with numerous vacuoles including autophagosomes in *Axl*^{-/-} mice during recovery (Hoehn et al., 2008). The degree of axonal damage in *Axl*^{-/-} mice was not as extensive as that seen in the DKO mice. Additionally, at 6-weeks cuprizone+6-weeks recovery the width of the corpus callosum of DKO mice was significantly narrower than WT mice suggesting that either fewer axons are present due to axonal dropout or fewer axons have properly remyelinated due to the preceding axonal damage. Therefore, while the axon's ability to efficiently recover from cuprizone toxicity is compromised in *Axl*^{-/-} mice, DKO are more severely affected.

In neurons and other cells in the CNS, constitutively active autophagy constitutes a major quality control mechanism contributing to the maintenance of cellular homeostasis (Boland et al., 2006; Kiffin et al., 2006; Levine, 2005; Nixon, 2013). Altered and dysregulated autophagy is commonly linked to neurodegeneration (Boland et al., 2006; Hara et al., 2006; Komatsu et al., 2006; Nixon, 2013), and dysregulated autophagy is observed in patients with MS (Alirezai et al., 2009; Liang et al., 2015). TEM analyses show that many of the axons in the DKO corpus callosum contain autophagosomes, autolysosomes, amphisomes and MVBs consistent with delayed or dysregulated autophagy. DKO mice provide a system to study this further.

Proper regulation and control of the inflammatory response is required to protect OLs from damage and to promote remyelination (Diemel et al., 1998; Marriott et al., 2008; Merson et al., 2010;). In demyelinating diseases such as MS, alterations in the innate immune system contribute to myelin damage, axonal loss and limit remyelination (Podbielska et al., 2013). We quantified the number of immune cells at the midline of the corpus callosum in both groups of mice and found no differences in the number of astrocytes and microglia/macrophages indicating that the recruitment of these glial cell populations are not affected by deletion of *Gas6*-*Axl* signaling. However, the proinflammatory cytokine profile in the DKO corpus callosum differed from WT during cuprizone treatment and withdrawal. In

response to cuprizone, DKO mice have increased proinflammatory mRNA expression and altered regulation of SOCS1 and SOCS3 expression, indicative of a lag in suppressing inflammation. In the WT corpus callosum, the upregulation of *SOCS1* mRNA expression at 5-weeks cuprizone likely serves as a protective response to IFN- γ signaling, in line with data demonstrating a protective role for SOCS1 against IFN- γ signaling in oligodendrocytes (Balabanov et al., 2006). DKO mice had a significant increase in IFN- γ expression and delayed remyelination, consistent with data showing that IFN- γ expression in the CNS suppresses remyelination in the cuprizone model and increases clinical scores during EAE (Lin et al., 2006). The inability of DKO mice to upregulate SOCS1 likely compromised the anti-inflammatory effects afforded by SOCS1 signaling. TNF- α has multiple functions depending upon its interaction with its receptors. TNF- α signaling is critical for OL regeneration when signaling through the TNFR-2 receptor (H.A. Arnett et al., 2001). As TNF- α is known to be a potent inducer of other proinflammatory molecules (Hurwitz et al., 1995), it is likely that the expression of TNF- α alone or in conjunction with dysregulated inflammatory signaling contributes to the phenotype seen in DKO mice in the cuprizone model.

Increased levels of several proinflammatory cytokines in the DKO mice suggest that ProS1/MerTK and/or Tyro3 signaling is insufficient in providing negative-regulatory signals to control neuroinflammation. This is reflected in the marginal increases in *Tyro3* and *MerTK* mRNA expression in DKO mice over the course of cuprizone diet and recovery, whereas WT mice showed *Tyro3*, *Axl*, and *MerTK* mRNA upregulation. Significant increases in ProS1 mRNA expression were observed in DKO mice in this study. The precise role of ProS1 in the CNS is not well defined. ProS1 was shown to aid in phagocytosis of apoptotic cells by microglia via MerTK signaling and aid in blood brain barrier (BBB) integrity via Tyro3 signaling (Fourgeaud et al., 2016; Zhu et al., 2010). During cuprizone diet, DKO mice showed highly elevated levels of *ProS1* mRNA expression, which should correlate with efficient debris clearance; yet, more Oil Red O+ deposits and lipid droplets by TEM were observed in DKO mice. A more pronounced delay in debris clearance following CNS insult was also observed in *Axl*^{-/-} mice (Hoehn et al., 2008; Weinger et al., 2011). As Gas6 seems to be primarily responsible for debris clearance, ProS1 upregulation may partially compensate for the loss of Gas6 signaling to aid in phagocytosis; however, it appears to not fully compensate during recovery. The presence of increased numbers of pathological lipid droplets in the DKO corpus callosum raises some intriguing questions since it is unclear whether these residual lipids are from the demyelinating process, defective lipid metabolism within glial cells, or lipid sources within oligodendrocytes that are required for remyelination.

Motor skill learning in mice requires the generation of new oligodendrocytes, expression of transcription factors and central myelination (McKenzie et al., 2014). Training and running on the wheel followed by testing on an uneven rung wheel was found to increase oligodendrocyte production and myelination (McKenzie et al., 2014). While wheel testing is informative for testing motor performance, examining remyelination following cuprizone withdrawal would be best using tests that do not require continual exercise over extended periods. Testing performance using balance beams and negative geotaxis does not require individual housing of mice, which can lead to depression, alter OPC number, or affect the

extent of remyelination relative to unexercised mice. DKO mice had significant behavioral defects at 5- and 6-weeks cuprizone treatment. At 1-week recovery, we observed no significant differences between DKO and WT mice in balance beam and negative geotaxis performance. As DKO mice have increased inflammation while on cuprizone diet with subsequent axonal damage at recovery time points, it is likely that uncontrolled inflammatory processes contributed to the observed behavioral deficits at 5- and 6-weeks cuprizone treatment.

Thus, the DKO mice provide an excellent model to further characterize functions attributed to Gas6 and Axl signaling in the CNS, and a means to test treatment paradigms in models where the DKO demonstrate prolonged inflammation, demyelination, and axonal deficits, pathologic aspects observed in individuals with MS.

Main Points

Deletion of Gas6-Axl signaling leads to prolonged neuroinflammation resulting in significantly more axonal damage and delayed remyelination in the cuprizone model. Mice lacking Gas6-Axl signaling have increased motor deficits while on the cuprizone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding sources include the National Multiple Sclerosis Society (NMSS) grants RG4046-A6 and RG5351-A-10; NINDS 1R21NS093134; IDRC Center Grant: P30HD071593; Shared Instrument Grant #1S10OD019961-01, Molecular Neuropathology Training Grant #T32NS007098. We thank Drs. Amanda Beck, DVM and Rani Sellers, DVM, Department of Pathology at Albert Einstein College of Medicine for performing necropsies on DKO and WT mice. We thank Dr. Asao Hirano, Professor Emeritus, Department of Pathology at Albert Einstein College of Medicine for sharing his knowledge of zebra bodies. (Hirano, 1971, 1997; Malamud et al., 1974). We thank Dr. John Alberta, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, for antibodies to Olig1 and Olig2. PDGFR α antibodies were generously provided Dr. William Stallcup, Sanford Burnham Prebys Medical Discovery institute, La Jolla, CA. We also acknowledge the technical help from Ms. Leslie Cummings and Ms. Xheni Nishku and advice we received from Mr. Frank Macaluso Director of the Einstein Analytic Imaging Facility.

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Figure 1.

Myelination is normal in naïve Gas6^{-/-}Axl^{-/-} (DKO) mice. A) DKO genotypes were confirmed by PCR and gel electrophoresis. The first lane in each panel shows the DNA molecular weight marker. Middle lane shows a lower migrating band signifying Gas6 (left panel) or Axl (right panel) knockout alleles; higher migrating bands signify the relevant WT alleles. All PCR products were evaluated on the same gel. B) 5µm sections of 4% paraformaldehyde fixed, paraffin-embedded brain and spinal cord sections were stained with H&E or MBP. Sagittal sections of brain denote the corpus callosum (arrows) of 8-week old WT (left) and DKO (right) and at higher magnification below. Equivalent MBP+ immunoreactivity is observed in cross sections of dorsal spinal cord. C) Equivalent numbers of cytoplasmic Olig1+ oligodendrocytes, and D) myelinated axons/X5000 TEM contact

sheets (n=3/group) in groups of mice; naïve male and female values were the same (presented as mean±SEM; student's t-test, p 0.05).

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Figure 2.

DKO mice have less remyelination and more axonal damage at 6-weeks cuprizone + 3-weeks recovery relative to WT mice. Aa–b,B) MBP immunostaining was performed on 4%-paraformaldehyde fixed paraffin-embedded sections. MBP content was determined by immunofluorescence and light microscopy (a,b). B) Mean fluorescence intensity (MFI) was determined using Image J software. DKO mice had a MFI of 4302 ± 168.4 (n=4) and WT mice had a MFI of 4977 ± 192.2 (n=3); student's t-test, p = 0.05. Ac–d,C) Cytoplasmic (arrows) and nuclear (arrow heads) Olig1+ cells are shown at 20x magnification (c–d). Supplemental Figure 1 shows the single and merged channels for DAPI and Olig1. Olig1+ cells were quantified by immunofluorescence at 60x magnification and translated to cells/mm². Significantly fewer DAPI+ cytoplasmic Olig1+ cells were seen in the DKO mice (591.3 ± 45 , n=11) relative to WT mice (739.7 ± 48.4 , n=9; Mann-Whitney U test, p=0.027) and had significantly more DAPI+ nuclear Olig1+ cells (202.4 ± 26.02 , n=7) relative to WT (86.0 ± 27.2 , n=6; Mann-Whitney U test, p=0.033). Ae–f,D) SMI32+ axonal swellings ($\geq 3\mu\text{m}$, arrows) were counted at 60x magnification via immunofluorescence. E) The mean number of SMI32+ axonal swellings ($\geq 3\mu\text{m}$, arrows) for DKO mice was $322.4/\text{mm}^2 \pm 74.5$ (n=6) and for WT mice was $138 \text{ swellings}/\text{mm}^2 \pm 32.7$ (n=7); student's t-test, p=0.036. Ag–h,E) APP+ axonal swellings ($\geq 3\mu\text{m}$, arrows) indicative of defective axonal transport were

quantified. E) The mean number of APP+ axonal swellings for DKO mice was 300.5/mm²±31.84 (n=6) and for WT mice was 156.4 swellings/mm²±39.4 (n=7); student's t-test, p=0.018. Significance for all counts was determined using GraphPad Prism v7 software with values presented as mean±SEM.

Figure 3.

TEM show extensive axonal pathology and fewer myelinated axons at 6-weeks cuprizone + 3- weeks recovery in male and female DKO mice. A) Transmission electron micrographs of WT (a–d) and DKO (e–g) mice show extensive axonal damage and pathology at the midline of the corpus callosum of DKO mice. DKO mice have more swollen axons (white arrows; Ae) containing multivesicular bodies (black arrows; Af,g), autophagosomes (black asterisk; Ag), and autophagolysosomes (white asterisk; Af,g) relative to WT mice. Boxes in Ab and enlarged in Ac,d show a rare enlarged unmyelinated axon in the WT corpus callosum (Ac), and a myelinated axon with vacuoles and damaged myelin (Ad). The number of myelinated axons in female and male DKO mice are significantly less than in WT mice (B,D). Also, the percentage of X5000 contact sheets/mouse containing swollen axons with MVBs and autophagic vacuoles was higher in DKO mice relative to WT mice (C,E). Values are present as mean±SEM. Statistical analysis was performed using a student's t-test in GraphPad Prism software.

Figure 4.

TEM show pronounced zebra bodies in the cell body of microglia and oligodendrocytes of DKO mice at 6-weeks cuprizone+3-weeks. Zebra bodies (black arrowhead) consisting of dark amorphous bodies composed of stacks of membranes were observed in WT (a) and DKO (b) corpus callosum. A large zebra adjacent to the nucleus of a microglia is seen in c; A denotes axon, M denotes microglia. The microglia is in contact with a swollen axon (white box) seen at higher magnification in d. A normal appearing oligodendrocyte with nucleus (lower right), rich cytoplasm and elongated process extending toward and contacting thinly myelinated axons indicative of remyelination is also observed in c (black box, enlarged in e). The arrow in e is indicative of a point of contact between the oligodendrocyte process and an axon at higher magnification. An oligodendrocyte with zebra bodies in the cell body adjacent to the nucleus is shown in f.

Figure 5.

DKO mice display defects in debris clearance and more pathological lipid droplets at 6 weeks cuprizone + 3 weeks recovery. A) Frozen sections from DKO and WT mice were incubated in Oil Red O to stain for neutral lipid debris during 5- and 6-weeks cuprizone and 6-weeks cuprizone + 1-week and 3-weeks recovery. Mounted slides were imaged on a Perkin Elmer P250 Panoramic Slide Scanner and images were analyzed in Panoramic Viewer software. B) Lipid droplets appearing as round gray spherical structures depicted by white arrowheads were counted in 5–7 contact sheets/mouse from male and female DKO and WT mice. DKO mice had significantly more lipid droplets at the midline of the corpus callosum following 3- weeks recovery from cuprizone. Values are presented as mean±SEM and analyzed using student's t-test.

Figure 6. Remyelination in DKO mice lags following 1-week and 3-weeks recovery from cuprizone diet. A) Immunofluorescent staining of OPCs (PDGFR α , left), Olig2+ OLs (center), and CC1+ OLs (right) at 5- and 6- weeks cuprizone diet and 1-week and 3-weeks recovery. No significant differences in the numbers of PDGFR α + OPCs, total Olig2+ OLs, and CC1+ OLs at the midline of the corpus callosum of WT (black bars) and DKO (grey bars) mice by Mann-Whitney U test. B) Frozen sections from WT and DKO mice after 5- and 6-weeks cuprizone and 6-weeks cuprizone + 1-week and 3-weeks recovery were stained with Myelin Black Gold-II (Millipore). C) Frozen sections from WT (n=9) and DKO (n=6) mice after 6-weeks cuprizone + 6- weeks recovery were stained as in B. Corpus callosum width displayed in micrometers $\pm$ SEM was measured using Panoramic Viewer software. Measurements were analyzed using in GraphPad Prism v7 software using student's t-test.

Figure 7.

Astrocyte and microglia/macrophage recruitment is not affected by deletion of Gas6-Axl signaling. Immunofluorescent staining for astrocytes (left, GFAP) and microglia/macrophages (right, Iba1) was performed on frozen sections (n=3–9/group/time point). GFAP+ and Iba+ glia were counted and translated to cells/mm²±SEM. Data were analyzed in GraphPad Prism by Mann-Whitney U test.

Figure 8.

DKO mice have increased pro-inflammatory cytokine expression at 6-weeks cuprizone treatment and 1-week recovery relative to WT mice. A 2-mm section of corpora callosa was isolated at the midline from DKO (gray thatched bars) and WT (black bars) mice following perfusion with PBS. The isolated region was homogenized in Trizol. Extracted RNA was reverse transcribed and qRT-PCR was performed. All genes were normalized to HPRT expression with naïve (0-weeks) WT mice set as the reference. Differences in expression are shown as 2^{-ddCt}. Data is presented as mean±SEM and analyzed in GraphPad Prism by student's t-test, *p* 0.05 as significant.

Figure 9.

Deletion of *Gas6* and *Axl* signaling causes behavioral and motor deficits in DKO mice during cuprizone-mediated toxicity. Two behavioral tests were used to assess motor function during cuprizone diet. A) Negative geotaxis was used to assess proprioceptive pathways and is measured by the latency (seconds) to right when placed head downward on a grid screen. Mice were tested after 5- and 6-weeks cuprizone diet. B) The balance beam test, assessing motor coordination as the number of slips made while crossing a round wooden beam over 3 levels of difficulty (with smaller diameter beams being harder). For A and B, data were analyzed in GraphPad Prism using Mann-Whitney, $p < 0.05$ as significant. C) Cognitive deficits in the object placement test visuospatial memory were significantly different between cuprizone-treated DKO mice and cuprizone-treated WT mice after 6-weeks when preference for the novel object is expressed as a preference score (left panel, $p=0.003$) or as the % of subjects passing and failing ($X^2=6.4$, $p<0.01$).

Table 1

qRT-PCR and Genotyping Primer Sets

Gene	Forward	Reverse
Axl	5'-ATGGCCGACATTGCCAGTG-3'	5'-CGGTAGTAATCCCCGTTGTAGA-3'
IFN- γ	5'-TCAAGTGGCATAGATGTGGAAGAA-3'	5'-TGGCTCTGCAGGATTTTCATG-3'
IL-1 β	5'-TGTAATGAAAGACGGCACACC-3'	5'-TCTTCTTTGGGTATTGCTTGG-3'
IL-6	5'-CAGAGGATACCACTACCAACAG-3'	5'-TCTCATTTCCACCACGATTTCCC-3'
Gas6	5'-GGGCCTAAAACTATCCCCAGA-3'	5'-GGTACAAGGACTTCACGCTCT-3'
HPRT	5'-AGTCCCAGCGTCGTGATTAG-3'	5'-TTTCCAAATCCTCGGCATAATGA-3'
MerTK	5'-CAGGGCCTTTACCAGGGAGA-3'	5'-TGTGTGCTGGATGTGATCTTC-3'
TGF- β 1	5'-ACCATGCCAACTTCTGTCTG-3'	5'-CGGGTTGTGTGGTTGTAGA-3'
TNF- α	5'-TGAACCTCGGGGTGATCGGTC-3'	5'-AGCCTTGTCCTTGAAGAGAAC-3'
ProS1	5'-CGCTTTTCGGGTGCTACTGG-3'	5'-CACTCTCGTTCAAGGTTGCC-3'
SOCS1	5'-CTGCGGCTTCTATTGGGGAC-3'	5'-AAAAGGCAGTCGAAGGTCTCG-3'
SOCS3	5'-ATGGTCACCCACAGCAAGTTT-3'	5'-TCCAGTAGAATCCGCTCTCCT-3'
Tyro3	5'-GCCTCCAAATTGCCCCGTCA-3'	5'-CCAGCACTGGTACATGAGATCA-3'
Genotyping Primers		
Gas6 (both)	5'-GAGTGCCGTGATTCTGGTC-3'	
Gas6 WT	5'-CCACTAAGGAAACAATAACTG-3'	
Gas6 KO	5'-ATCTCTCGTGGGATCATT-3'	
Axl (both)	5'-AGAAGGGGTTAGATGAGGAG-3'	
Axl WT	5'-GCCGAGGTATAGTCTGTCACAG-3'	
Axl KO	5'-TTTGCCAAGTTCTAATTCCATC-3'	

Measurements (presented as micrometer±SEM) and g-ratios in naïve and 6-weeks cuprizone diet + 3-weeks recovery in female DKO and WT mice determined using Image J software.

Table 2

Genotype	Treatment status	Axonal Diameter	Myelin Thickness	Axonal Diameter + Myelin Thickness	g-ratio
WT	Naïve	0.729±0.088	0.250±0.044	0.981±0.100	0.739±0.017
	6 weeks + 3 weeks	0.735±0.056	0.184±0.004	0.919±0.058	0.788±0.011
DKO	Naïve	0.700±0.051	0.203±0.013	0.902±0.049	0.764±0.017
	6 weeks + 3 weeks	0.820±0.061	0.179±0.010	0.999±0.063	0.813±0.014

Table 3

Observations in $Axl^{-/-}$, $Gas6^{-/-}$ and DKO mice relative to WT during cuprizone-mediated toxicity and recovery.

	DKO	$Axl^{-/-}$	$Gas6^{-/-}$
Myelin Debris Clearance	Impaired debris clearance	Lag in myelin debris clearance ¹	N/D [*]
Axonal Damage	Increased SMI32+ and APP+ swollen axons with numerous MVBs and autophagosomes	Increased SMI32+ and APP+ swollen axons ¹	N/D [*]
Number of CC1+ Oligodendrocytes During Recovery	No significant difference in the number of CC1+ oligodendrocytes at 3-weeks recovery; significant differences in nuclear vs. cytoplasmic Olig1	No significant difference in the number of CC1+ oligodendrocytes at 3-weeks recovery ¹	Fewer CC1+ oligodendrocytes after 4-weeks recovery ³
Glial Recruitment at the Corpus Callosum	Equivalent Iba1+ microglia/macrophages and GFAP-positive astrocytes	Equivalent Iba1+ microglia/macrophages ¹	More Iba1+ microglia/macrophages during cuprizone toxicity and recovery ²
Inflammatory Response in the Corpus Callosum	Increased pro-inflammatory cytokine mRNA expression post-cuprizone withdrawal	N/D	N/D
Behavioral Deficits	Increased motor and cognitive deficits	N/D	N/D
Myelination	Decreased and delayed remyelination, fewer myelinated axons, no difference in g-ratio or myelin thickness	No difference in g-ratio or myelin thickness ¹	Decreased remyelination, fewer myelinated axons, no difference in g-ratio or myelin thickness ^{2,3}
Additional Pathology in the Corpus Callosum	Large zebra bodies and lipid droplets	N/D	N/D

^{*} Not Discussed (N/D) in the publication;

¹ (Hoehn et al., 2008);

² (Binder et al., 2008);

³ (Binder et al., 2011)