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The Anti-Aging Protein Klotho Enhances Remyelination Following Cuprizone-Induced Demyelination

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Abstract The current study examined whether overexpression of Klotho (KL) in transgenic mice can enhance remyelination following cuprizone-induced demyelination and improves the clinical outcome in experimental autoimmune encephalomyelitis (EAE). Demyelination was achieved by feeding transgenic mice overexpressing the transmembrane form of Klotho (KL-OE) and wild-type (WT) littermates cuprizone-containing chow for 6 weeks. The animals were then allowed to remyelinate for 3 weeks. Paraphenylenediamine staining and platelets-derived growth factor receptor α (PDGFRα) and glutathione S-transferase pi (GSTpi) immunohistochemistry were performed on corpus callosum (CC) sections for quantification of myelin and progenitor and mature oligodendrocytes, respectively. The EAE model was induced with the $MOG_{35–55}$ peptide. The animals were scored daily for clinical symptoms for 30 days. Following 6 weeks of demyelination, both KL-OE mice and WT littermates demonstrated almost complete and comparable demyelination of the CC. However, the level of spontaneous remyelination was increased approximately two-fold in KL-OE mice, although no significant differences in the numbers of PDGFRα and GSTpi-positive cells were observed. Following EAE induction, Klotho overexpression did not affect the clinical scores, likely due to the different roles Klotho plays in the brain and spinal cord. Thus, increasing Klotho expression should be considered as a therapy for enhancing remyelination in the brains of individuals with multiple sclerosis.

Keywords Multiple sclerosis · Oligodendrocyte differentiation · Myelin repair · Small molecule compounds

Abbreviations

CC	Corpus callosum
CSF	Cerebrospinal fluid
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular regulated kinase
GSTpi	Glutathione S-transferase pi
KL	Klotho
KL-OE	Klotho overexpressing
KL-KO	Klotho knockout
MOG	Myelin-oligodendrocyte glycoprotein
MS	Multiple sclerosis
OPC	Oligodendrocyte progenitor cell
OL	Oligodendrocyte
PDGFRα	Platelets-derived growth factor receptor α
PLP	Proteolipid protein
PPD	Paraphenylenediamine
sKL	Shed Klotho
TNF-α	Tumor necrosis factor α
WT	Wild type
IP	Intraperitoneal

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Introduction

Multiple sclerosis (MS) is a chronic central nervous system (CNS) demyelinating disorder, with main features of central inflammation and progressive destruction of myelin and axons. All pharmacological agents aimed to overcome MS had been targeting an overactive immune response. However, enhancing the incomplete remyelination found in MS lesions presents another potential target for the development of new therapeutic approaches for the disease. The generation of new oligodendrocytes (OLs) and myelin are prominent features of white matter lesions during early stages of MS Prineas et al. (1993). The reduced recruitment of oligodendrocyte progenitor cells (OPCs), insufficient generation of OPCs into OLs, and failure of OL differentiation into mature myelin forming cells were shown to be the prominent contributors to chronically demyelinated white matter lesions in MS brains (Chang et al. 2000; Chang et al. 2002; Kuhlmann et al. 2008; Chang et al. 2012). Remission is mainly achieved by migration of OPCs to sites of injury and their subsequent maturation into myelin-producing cells, while the inhibition of OPC differentiation at sites of lesion was recognized as a major contributor to disease progression (Trapp et al. 1998; Chang et al. 2002).

Although MS is exclusively a human disease, several animal models for the disease have been established. The cuprizone animal model of toxic demyelination is utilized to examine different processes reflecting demyelination and remyelination in the CNS (Torkildsen et al. 2008). Apoptosis of primary OLs together with activated microglia present two major histopathological features of the cuprizone model. These hallmarks are associated with human pathology in type 3 and type 4 MS lesions (MS3 and MS4) (Lucchinetti et al. 2000; Barnett and Prineas 2004), in which oligodendrogliopathy is a main characteristic of pattern 3 and primary oligodendrocyte damage with secondary demyelination is associated with pattern 4 (Lucchinetti et al. 2000). Two other MS patterns (MS1 and MS2) closely resemble T cell-mediated or T cell plus antibody-mediated autoimmune encephalomyelitis, respectively (Lucchinetti et al. 2000), (reviewed in (Kipp et al. 2009). In experimental myelin-oligodendrocyte glycoprotein (MOG)-induced autoimmune encephalomyelitis (EAE) animal model of MS (Ben-Nun et al. 2014; Ellwardt and Zipp 2014), the disease is induced through T cell-mediated CNS demyelination and is a valid tool for studying immune-mediated type of demyelination, associated with pattern 1 and 2 of MS.

The anti-aging protein Klotho (KL) was named after the goddess of Fate from the Greek mythology that is "responsible for spinning the thread of human life" (Kuro-o et al. 1997). Among other organs, KL is primarily expressed in the kidney and in the brain (Kuro-o et al. 1997). KL is a type I transmembrane protein that undergoes ectodomain shedding by ADAM10 and 17 (Chen et al. 2007; Bloch et al. 2009). Cleaved (soluble) form of KL (sKL) is detectable in serum and cerebral spinal fluid (CSF)(Imura et al. 2004). The extracellular domain of Klotho contains two regions that exhibit homology with glycosidases. In the kidney, the full-length, transmembrane KL (FL-KL) acts as a co-receptor with Fibroblast growth factor receptor 1 (FGFR1) for FGF23 and participates in the regulation of serum levels Vitamin D and phosphate. Circulating KL was shown to play a role as a hormone/ligand and/or a glycosidase in maintaining ion homeostasis and in suppressing the Wnt and insulin/IGF1 signaling pathways (Kuro-o 2010).

In the brain, KL is produced mainly by the ependymal cells of the choroid plexus and by hippocampal neurons (Kuro-o et al. 1997). KL is also detectable in cerebral white matter (Duce et al. 2008) and in the pituitary gland (Li et al. 2004). Mice that lack KL (KL knockout, KL-KO) develop specific phenotype changes that resemble human aging, such as arteriosclerosis, osteoporosis, and cognitive decline. These mice develop normally, albeit smaller than wild-type littermates, until 3 to 4 weeks of age when they begin to demonstrate growth retardation and die prematurely at an average age of ~61 days (Kuro-o et al. 1997). In contrast, the lifespan of KL overexpressing (KL-OE) mice is 30 % longer than that of wild-type (WT) mice (Kurosu et al. 2005). KL-KO mice exhibit learning and memory deficits that appear in early adulthood (Nagai et al. 2003), a reduced number of synapses in the hippocampus (Li et al. 2004), axonal transport disturbances, and neurodegeneration of the hippocampus (Shiozaki et al. 2008). We have showed recently that a lifespan-extending polymorphism of the human Klotho gene, KL-VS, correlates with enhanced cognition in heterozygous carriers. Furthermore, KL-OE mice performed better in learning and memory tasks. This effect was mediated through the enrichment of the synaptic GluN2B subunit of the N-methyl-D-aspartate receptor (NMDAR) (Dubal et al. 2014). Moreover, we provide evidence that KL is able to counteract cognitive deficits and premature mortality in human amyloid precursor protein (hAPP) transgenic mice J20, a well-characterized animal model of Alzheimer disease (Dubal et al. 2015). In vitro, we have demonstrated the neuroprotective properties of KL on hippocampal neurons against glutamate and amyloid beta peptide cytotoxicity through an increased resistance to oxidative stress (Zeldich et al. 2014).

Our previously performed microarray analyses aimed at revealing factors associated with age-induced cognitive decline, demonstrated decreased expression of KL in the aged corpus callosum of the rhesus monkey (Duce et al. 2008). The decreased expression was due, at least in part, via the hypermethylation of the KL promoter (King et al. 2012a). Decreased levels of KL correlate with ubiquitous dysmyelination observed in the brains of aged monkeys by us as well as by other groups (Sloane et al. 2003; Hinman et al. 2006; Hinman and Abraham 2007; Makris et al. 2007; Wisco

et al. 2008; Bowley et al. 2010; Kohama et al. 2012). In support of a possible involvement of KL in myelination of the CNS, we have reported that KL-KO mice exhibited severely impaired myelination of the optic nerve and corpus callosum accompanied by significant abnormalities at the nodes of Ranvier. In vitro, we have found that KL promotes maturation of OPCs into myelin-producing OLs via extracellular regulated kinase (ERK) and Akt signaling pathways (Chen et al. 2013). In addition, a recent study showed decreased concentrations of KL in the CSF of patients with relapsing-remitting multiple sclerosis (Emami Aleagha et al. 2015).

In light of the recent discovery pointing out to the prominent role KL plays in enhancing cognition, neuroprotection, and myelination, more studies are required to further characterize the effect of KL in the CNS and its clinical relevance to neurological disorders, such as MS.

The aim of the current study was to assess whether overexpression of KL in transgenic mice affects demyelination and remyelination in the cuprizone model and improves the clinical score in the MOG-induced EAE animal model of MS. A positive outcome would indicate that increasing Klotho expression could enhance remyelination in demyelinating diseases of the CNS and may improve the neurological outcome.

Methods

Animals

C57BL/6 J mice were obtained from Charles River Laboratories and housed in the AAALAC accredited Boston University School of Medicine animal facility, and all studies were conducted under the regulations required by NIH and the BUSM IACUC committee. KL-OE mice (Kuro-o et al. 1997; Kurosu et al. 2005) were a kind gift from Dr. M. Kuro-o (UT Southwestern Medical Center, TX and Jichi Medical University, Japan) and were generated as described by microinjection of a linearized construct that expresses mouse KL cDNA driven by human elongation factor EF-1α promoter thought to be ubiquitously active at all developmental stages.

Genotyping

At the day of weaning, the tail from each mouse was clipped into a microcentrifuge tube and genomic DNA was isolated according to the manufacturer's protocol using the DirectAmp™ Tissue Genomic DNA Amplification Kit (Denville Scientific, Metuchen, NJ). Briefly, 100 μL of extraction solution was added to the tail followed by 25 μL of preparation solution. The samples were incubated at RT for 10 min and then at 95 °C for 3 min. One hundred microliter of neutralization solution was added and samples were mixed by vortexing. Four microliter of the tissue extract was used for PCR amplification using Choic Taq Blue Mastermix (Denville Scientific, Metuchen, NJ) in a 20 μL final volume. The Klotho primers used were: 5′- TCGCGCCCTGGCCG ACCATTTCAGG-3′ and 5′- AGCACAAAGTCAAGAG ACTTCTGGC-3′. PCR was carried out using the following cycling conditions: 94 °C for 3 min and 25 cycles at 94 °C 30 s, 58 °C 30 s, and 68 °C 30 s, followed by 68 °C 2 min. The PCR products were analyzed by 1 % agarose gel electrophoresis for 15 min. Samples from Klotho overexpressing mice produce a 339 bp band while the wild-type samples produce no band.

Induction of Cuprizone Model

Ten-weeks-old heterozygous KL-OE males and WT littermates were used in this study. Briefly, cuprizone (0.3 % (*w*/*w*), was mixed into chow pellets by Harlan Teklad (Madison, WI) which was available ad libitum for 6 weeks. During these 6 weeks, the animals received daily injections of rapamycin (ip, 10 mg/kg) to inhibit OPC proliferation. Body weights were recorded on a weekly basis. Starting from week 7, cuprizone-containing chow was replaced with normal diet and rapamycin injections were discontinued to permit remyelination. The animals were allowed to remyelinate for 3 weeks.

Induction of Experimental Autoimmune Encephalomyelitis (EAE)

Heterozygous KL-OE C57BL/6 J females and WT littermates age 8–12 weeks were immunized by subcutaneous injections with 300 μg of the $MOG_{35–55}$ peptide in CFA (Difco) with 500 μg M tuberculosis (H37RA; Difco). Mice received intraperitoneal injections of 400 ng of pertussis toxin (List Biological Laboratories, Campbell, California) the same day and again 48 h later. The animals were monitored daily for clinical symptoms of EAE, graded on the scale 0 = healthy, 1 = flaccid tail, 2 = ataxia and/or paresis of hindlimbs, 3 = paralysis of hindlimbs and/or paresis of forelimbs, 4 = tetraparalysis, 5 = moribund.

Transcardial Perfusion and Tissue Processing

Cuprizone Model To harvest tissue for histological analysis, all mice were anesthetized with 100 μL ketamine/xylazine and transcardially perfused with buffered 4 % paraformaldehyde (PFA). Brains were removed, and a coronal slice (approximately 1 mm thickness) was cut using a custom-made brain-slicing mold. The slice was then post-fixed for 2–3 days in 2.5 % glutaraldehyde/4 % PFA fixative in 0.4 M Sorenson's phosphate buffer (7.176 g/L of NaH_2PO4, 49.4 g/L of Na_2HPO4, pH-7.4) before embedding in Epon. The remaining rostral and caudal brain parts were placed in 4 % PFA in 0.4 M

Sorenson's phosphate buffer for 24–48 h at 4 °C and transferred to cryoprotection solution at 4 °C until completely infiltrated. Cryoprotected hippocampal tissue was sectioned on a freezing microtome to make 30 μm free-floating sections. Selected slices were immunostained with proteolipid protein (PLP), platelets-derived growth factor receptor α (PDGFRα), and glutathione S-transferase pi (GSTpi) antibodies to quantify myelin, OPCs, and OLs, respectively, in the corpus callosum (CC).

EAE Model The spinal cord was removed from spinal column by removing the vertebrae. Each region of the spinal cord (cervical, thoracic, and lumbar) was sliced into alternating 3- and 1-mm sections. The 1-mm sections of the tissue were post-fixed in 2.5 % glutaraldehyde/4 % PFA for Epon embedding, and the 3-mm sections of the tissues were post-fixed in 4 % PFA for 24–48 h, cryoprotected and stored at 4 °C for immunohistochemical analysis.

Epon Embedding, Sectioning, and Paraphenylenediamine Staining

Quantification of myelinated axons was processed as previously stated (Estable-Puig 1965; Trapp et al. 1981; Schaechter and Sadun 1985; McGavern et al. 2000; Sachs et al. 2014). Briefly, the tissue samples were washed for 3 × 10 min in 0.08 M Sorenson's buffer and placed in 2 % osmium tetroxide (OsO_4) for 2 h at room temperature. The tissues were then washed twice in 0.08 M Sorenson's buffer and dehydrated through graded alcohols: 70, 80, 95 % EtOH (2 × 10 min each) and 100 % EtOH (3 × 15 min) at room temperature in a fume hood on a rotator. The tissues were then washed twice with polypropylene oxide (2 × 10 min) and then placed in 1:1 polypropylene oxide/Epon mix and rotated overnight in an uncapped tube in the fume hood. The tissues were placed in fresh Epon for 1 h on the rotator and then embedded in a mold and baked at 60 °C for 24 to 48 h. Epon blocks were faced by cutting a number of 1–2 μm sections until the block face was smooth and all mechanical damage was removed. A total of 15–20 slides with 5–6 semi-thin (1 μm) sections on each slide were generated per animal. For the analysis of myelinating axons, two slides were stained with paraphenylenediamine (PPD) for 15 min and rinsed three times in absolute ethanol. The slides were then rinsed three times in xylene, air-dried, and coverslipped for analysis.

Immunohistochemistry

Quantification of myelination, OPCs, and OLs were processed as previously described (Dutta et al. 2011; Chang et al. 2012; Sachs et al. 2014). Briefly, free-floating sections (30 μm) of the corpus callosum were stained with antibodies to PLP (Cleveland Clinic Hybridoma), PDGFRα (Cell Signaling), and GSTpi (Assay Designs) to quantify myelin, OPCs, and OLs, respectively.

Microscopic Evaluation

Epon-PPD-stained sections of CC were evaluated in bright field at 10, 20, and 63× (oil objective). A total of 4 images (63×) per animal were collected in the region of interest (ROI). The images outside the ROI were not scored. The images were then evaluated using NIH ImageJ to identify and count myelinated axons in the ROI. The average number of PPD-stained myelinated axons per unit area in an anatomically defined region of the corpus callosum was then used for statistical calculations. Whole sections stained with anti-PLP were scanned in a Mirax slide scanner with a 20× lens (NA 0.8). The images were then exported at 10× for further processing using NIH ImageJ software (Fiji) and proprietary algorithms (Renovo Neural, Inc.). PLP stained sections were evaluated to assess myelin in hippocampus. To quantify the extent of myelination, hippocampi were outlined, thresholded (to isolate PLP staining from the background), and the percentage of areas occupied by PLP staining over the ROI was calculated with Image J. The percentages of myelin from the two hippocampal regions of the same slice were averaged, and the numbers from two brain slices were averaged again to obtain the result for each animal. All thresholded images were overlaid over the original images to confirm that thresholding reflects original PLP staining. PDGF and GSTpi stained slides were evaluated to assess OPC and OL cell number in the CC, respectively. To quantify the cell numbers, CC was outlined, and density of the cells was processed using the NIH Image J software and a proprietary algorithm as above.

Statistical Analysis

Quantitative data are expressed as the means ± SE. Statistical comparisons between experimental groups were made using the two-tailed, unpaired Student's *t* test. Probability values of $p < 0.05$ were considered significant.

Results

The Cuprizone Model

Under naïve (no demyelination by cuprizone) conditions, the number of myelinated axons was similar in CC of KL-OE mice and their WT littermates (Fig. 1a, b, g), suggesting that overexpression of Klotho does not affect the number of myelinated axons during development. Demyelination was induced by cuprizone chow plus daily ip rapamycin injections for 6 weeks. Rapamycin is used to inhibit OPC differentiation during the demyelination phase to achieve a clean/low

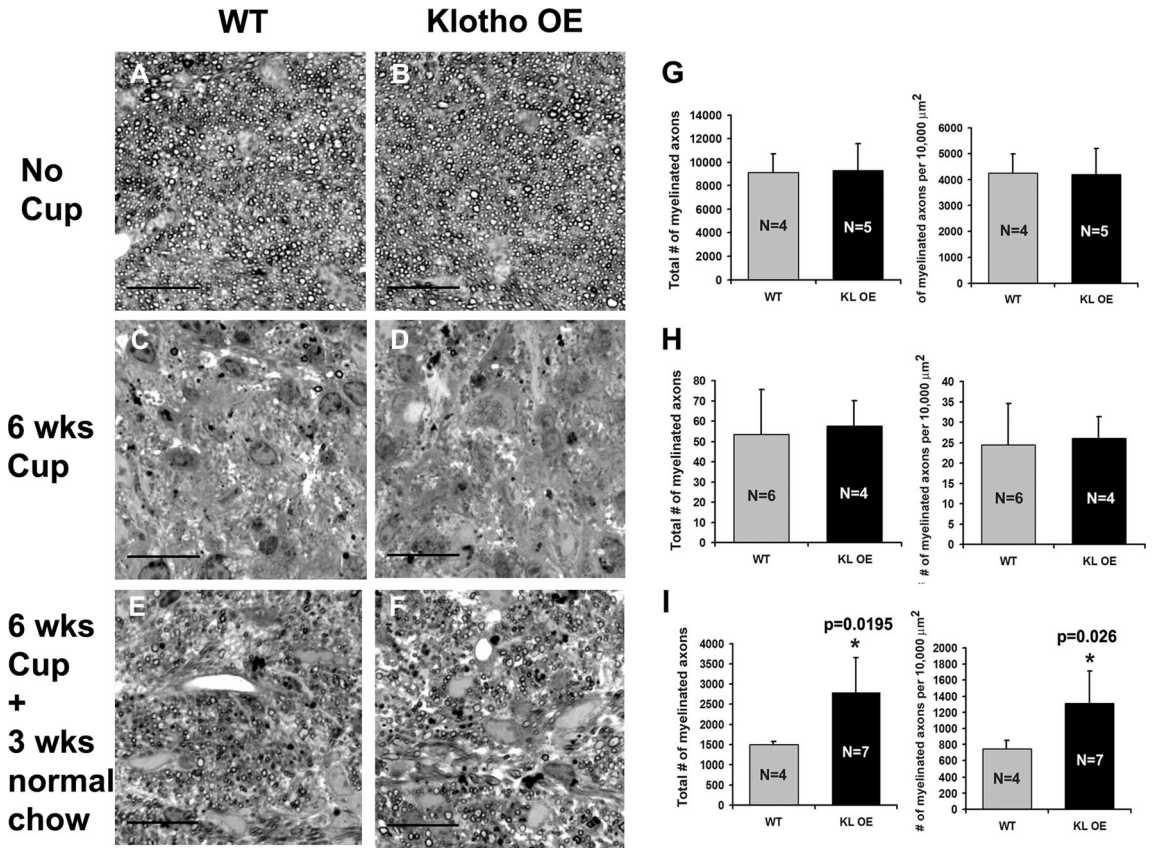

Fig. 1 Representative PPD staining images at 63× magnification from CC. **a**, **b** Animals that were exposed to naïve, non-demyelination conditions. **c**, **d** Animals that received 6 weeks of cuprizone to induce demyelination. **e**, **f** Animals that were exposed to 6-weeks of demyelination followed by 3-weeks of remyelination. **g–i** Statistical analysis of the total number of myelinated axons and the density of myelinated axons from **a** and **b**, **c** and **d**, and **e** and **f**. The results are presented as mean ± SE; *p* value from *t* test and number of mice used. *Cup* cuprizone. *Scale bar* = 10 μm

baseline for demyelination (Dutta et al. 2013). Following 6 weeks of the demyelination regimen, KL-OE mice and WT littermates demonstrated almost complete demyelination (less than 1 % of naïve animals) in CC when assessed by PPD staining in semi-thin (1 μm) tissue sections. The total number of myelinated axons was reduced from ~9000 under naïve conditions to ~60 under the demyelination conditions, and the density was reduced from ~4000/10,000 μm^2 to ~25/10,000 μm^2 (Fig. 1c, d, h). A subset of demyelinated animals was allowed to spontaneously remyelinate (normal chow and no rapamycin) for 3 weeks (Fig. 1e, f). The number of myelinated axons in KL-OE mice per unit length of the CC was 1.88 times higher ($p = 0.019$) than in WT animals (Fig. 1i). In addition, the density of myelinated axons per unit area increased by 1.76-fold ($p = 0.026$) in the KL-OE animals (Fig. 1i). These data indicate that Klotho overexpression significantly increased remyelination in the CC when compared to WT littermates.

Next, we assessed the potential remyelinating effect of Klotho overexpression in the hippocampus. We found no difference in the percentage of myelinated axons in hippocampus of KL-OE mice when compared to WT littermates, as assessed by PLP staining under naïve conditions (Fig. 2a, b, g). Under the demyelinating conditions, induced by the cuprizone diet, the percent area occupied by PLP-positive structures in the hippocampus was reduced from ~50 % to ~18 % (Fig. 2g, h), but no difference in demyelination was observed between KL-OE and WT animals (Fig. 2c, d, h). In contrast to the effect of Klotho overexpression in CC, the major white matter tracks connecting the left and right hemispheres, on remyelination, no difference in the remyelinating potential was found in the hippocampus of KL-OE animals vs. their WT littermates (Fig. 2e, f, i), suggesting that KL overexpression did not improve remyelination in the hippocampus, a gray matter structure.

In an attempt to understand whether the beneficial effect of KL overexpression on remyelination in the CC is due to more mature OLs, we quantified OPCs and mature OLs in KL-OE and WT mice by immunohistochemistry with PDGFRα and GSTpi staining, respectively, after the animals were allowed to remyelinate for 3 weeks. As shown in Fig. 3a–c, no statistical difference was found in PDGFRα cell density between KL-OE and WT animals, suggesting that Klotho effect is not mediated through the increased number of OPCs. Similarly,

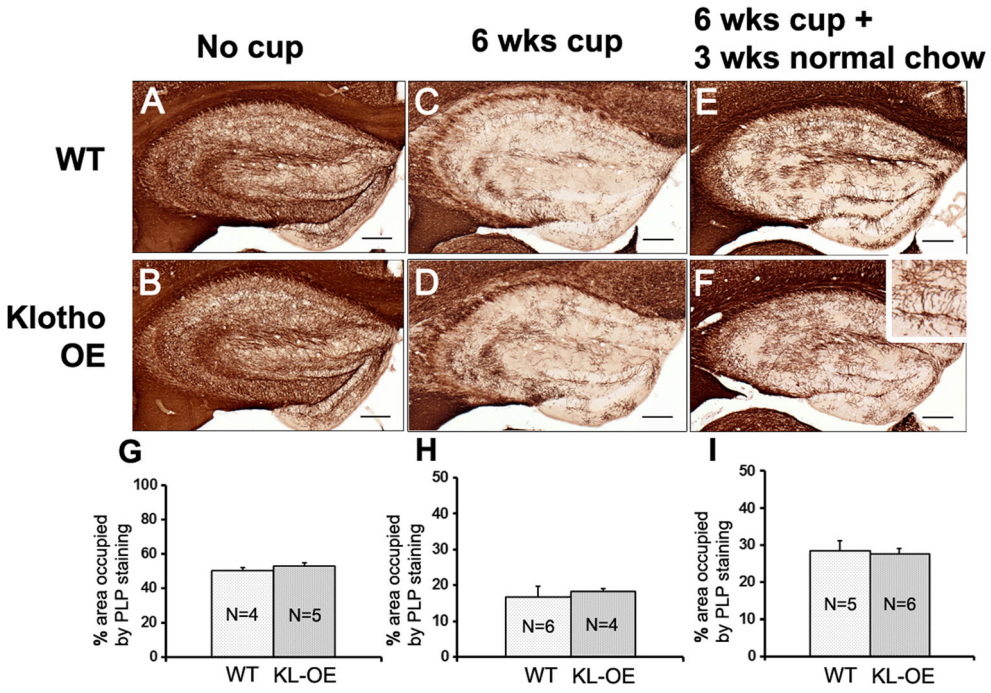

Fig. 2 Representative PLP staining images at 10× magnification of free-floating sections of hippocampus obtained from WT and KL-OE mice. Quantitative analysis of the extent of myelination in hippocampi calculated as the percentage of areas occupied by PLP staining over the region of interest (*ROI*). **a**, **b** Animals that were exposed to naïve (non-demyelination conditions). **c**, **d** Animals that received 6 weeks of cuprizone demyelination. **e**, **f** animals that were exposed to 6 weeks of demyelination followed by 3 weeks of remyelination; inset is a higher resolution image representing individual myelinated axons. **g**–**i** Statistical analysis of the percentage area occupied by PLP staining from **a** and **b**, **c** and **d**, and **e** and **f**. The results are presented as mean ± SE; *p* value from *t* test. Number of mice used per group is shown. Naïve: WT $n = 4$, KL-OE $n = 5$; 6 weeks of cuprizone: WT $n = 6$, KL-OE $n = 4$; 6-weeks cuprizone followed by 3-weeks of remyelination: WT $n = 5$, KL-OE $n = 6$. *Cup* cuprizone. *Scale bar* = 100 μm

KL overexpression had no effect on GSTpi + cell density in CC, implying that there is no difference in the number of mature OLs between the groups (Fig. 3d, e, f).

The EAE Model

EAE was induced in KL-OE and their WT littermates by $MOG_{35–55}$ (Adelmann et al. 1995; Ellwardt and Zipp 2014). Animals were monitored daily for clinical symptoms and scored. All immunized animals developed the disease at the same (97 %) rate of incidence and with no significant difference in the mean day of the onset of the disease that was between 10th to 11th day post-induction with the mean peak of disease severity of 3, as was scored using the scale 0–5, as mentioned in the materials and methods. Between the day of induction (day 0) and day 30, all mice in both groups developed clinical deficits corresponding to a score of grade 2 or greater. Our results revealed no difference in clinical pathology (Fig. 4a) between KL-OE and their WT littermates in the time frame of 30 days post-induction of the disease. Overall weights also did not differ between KL-OE and their WT littermates (Fig. 4b).

Discussion

The current standard of care for MS includes several anti-inflammatory and immunomodulatory drugs that promote clinical benefit by modulating inflammatory/immune responses. While these therapies can inhibit the progression of the disease and the clinical symptoms, they are unable to promote or induce remyelination of the demyelinated axons. Although the CNS responds to myelin destruction by inducing developmental mechanisms aimed at triggering remyelination, the endogenous repair is insufficient in patients with MS and this failure of remyelination is one of the leading contributors to neurological deficits in demyelinating disorders (Trapp et al. 1998; Chang et al. 2002).

In the current study, we present a comprehensive approach toward understanding the role Klotho plays in demyelination and remyelination processes using two well-characterized animal models for studying MS: the cuprizone and EAE models. Here, we demonstrate, for the first time, the remarkable effect of Klotho on the remyelination process in KL-OE mice following cuprizone demyelination. While the level of cuprizone-induced demyelination was similar in KL-OE and WT animals, the extent of spontaneous remyelination was

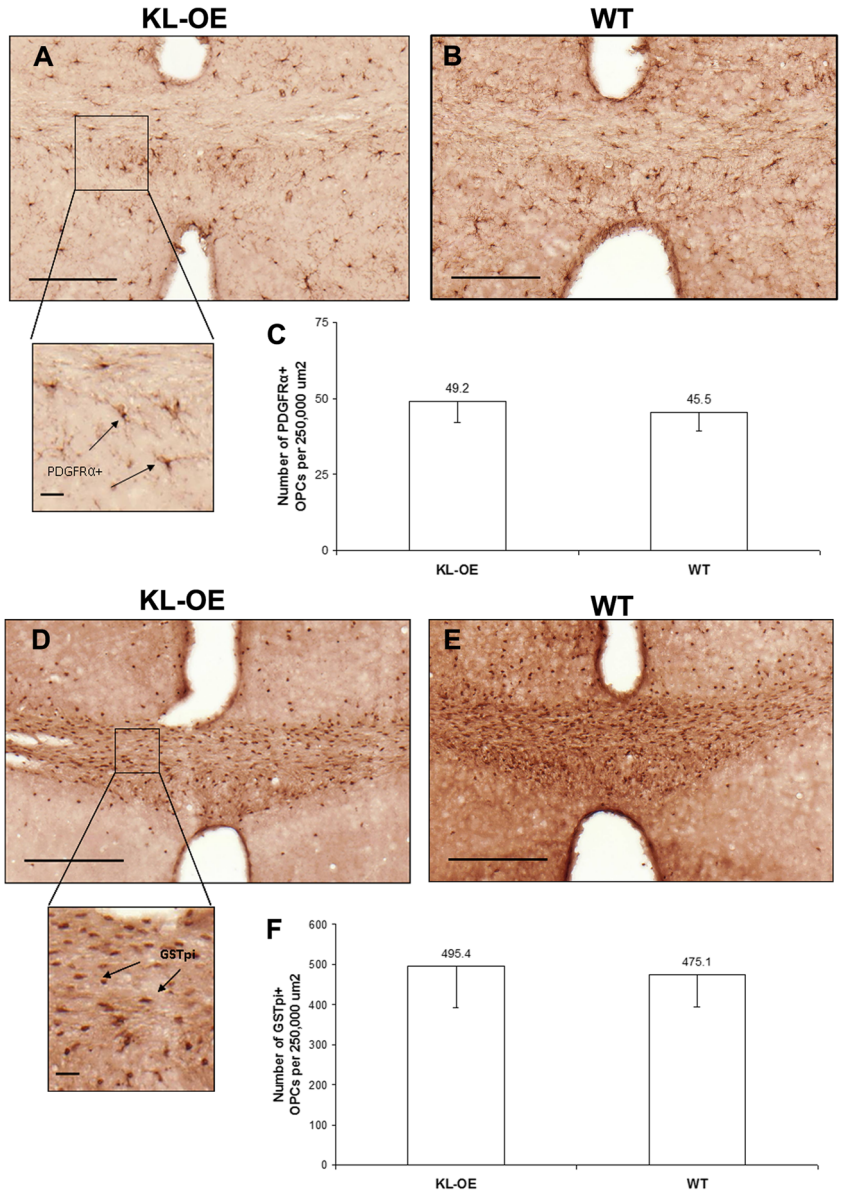

Fig. 3 Representative PDGFα (**a**, **b**) and GSTpi (**d**, **f**) staining images at 20× magnification of 30 μm floating sections of CC obtained from WT and KL-OE mice that were exposed to 6 weeks of demyelination followed by 3 weeks of remyelination. **c**, **f** Statistical analysis of PDGFRα+ and GSTpi+ cells represented as the density of cells within the CC from **a** and **b** and **d** and **f**, respectively. The results are presented as mean ± SE. *p* value from *t* test and number of mice used are shown (for PDGFRα and GSTpi analysis (WT $n = 5$, KL-OE $n = 7$). *Cup* cuprizone. *Scale bar* = 100 μm

increased almost two-fold in the white matter of KL-OE mice, strongly supporting the important role Klotho plays in accelerating remyelination. Interestingly, although KL-KO mice have severe hypomyelination (Chen et al. 2013), naïve KL-OE mice have similar numbers of myelinated fibers as the WT mice. In contrast to the positive results obtained using the cuprizone model, KL overexpression had no effect on the clinical scores in the MOG-induced EAE model. The negative EAE results were somewhat surprising because others found that Klotho possesses anti-inflammatory properties, although those studies were not conducted in the brain (Maekawa et al. 2009; Thurston et al. 2010).

In our previous studies, we reported a correlation between cognitive decline and decreased expression levels of KL protein and reduced myelin integrity in the brain white matter of aged monkeys (Sloane et al. 2003; Hinman et al. 2004; Duce et al. 2006; Hinman and Abraham 2007; Duce et al. 2008). We also observed a prominent reduction of myelinated fibers, as well as decreased numbers of OLs in the corpus callosum and optic nerves of KL-KO mice (Chen et al. 2013). In vitro, the exogenous addition of sKL fosters the maturation of OPCs (Chen et al. 2013). Recently, decreased levels of KL were found in the CSFs of MS patients (Emami Aleagha et al. 2015). Finally, others and we reported on the neuroprotective properties of KL (Kuang et al. 2014; Zeldich et al. 2014).

The cuprizone model of demyelination is widely used since the demyelination can be triggered in a very reproducible manner, and the formation of lesions is highly predictable (Kipp et al. 2009). Another benefit of using this model is that the demyelination is reversible allowing for the opportunity to study the remyelination process (Kipp et al. 2009). In addition, cuprizone-induced demyelination partially mimics

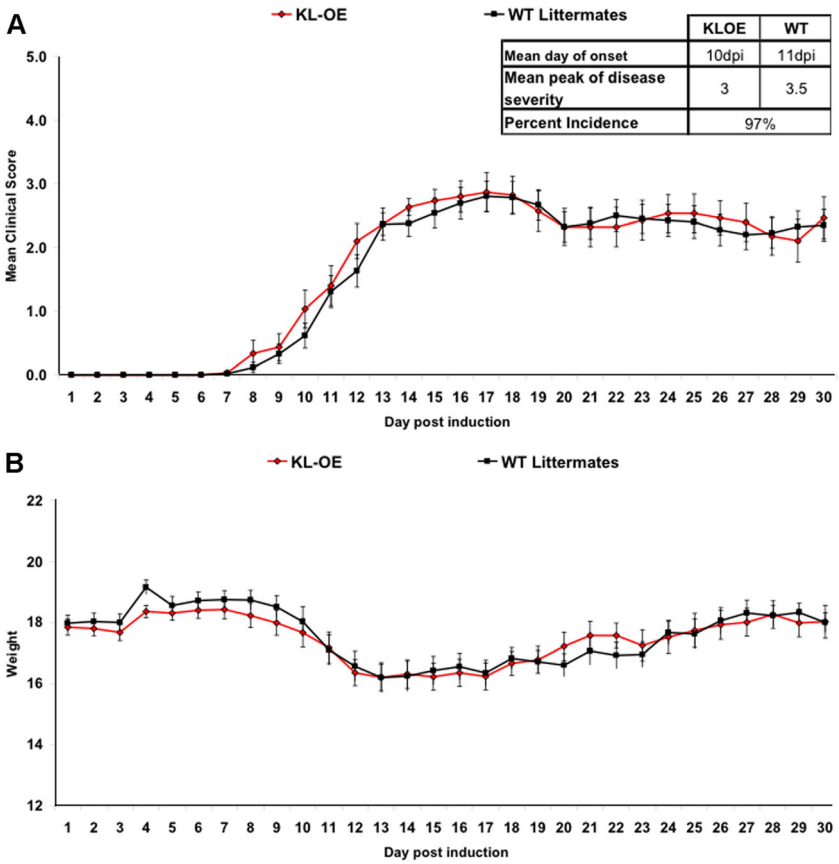

Fig. 4 Assessment of EAE clinical scores (**a**) and average weight (**b**) performed on KL-OE mice ($n = 15$) and their WT littermates ($n = 26$) at 0–30 days post-injection (*dpi*). The results are presented as mean ± SE

pathological steps of newly forming MS lesions in humans, associated with the lesions in type 3 and 4 MS (Lucchinetti et al. 2000). Cuprizone-induced demyelination occurs through OL death and, therefore, can be distinguished from the demyelination triggered by the involvement of the peripheral immune system (Kipp et al. 2009). In contrast, lesions in type 1 and 2 MS are associated with T cell-mediated immune attack (Lucchinetti et al. 2000) and the EAE mouse model is more experimentally approachable for studying the autoimmune mechanism of the disease.

The opposite correlation between KL expression and inflammatory responses was demonstrated previously. KL is down-regulated in the healthy elderly and especially in rheumatic arthritis with CD4+ lymphocytes (Witkowski et al. 2007). KL suppresses TNF-α-induced expression of adhesion molecules and NF-κB activation in human umbilical vein endothelial cells (Maekawa et al. 2009). KL expression is inhibited in mouse models of inflammatory bowel disease and is transcriptionally regulated by the pro-inflammatory cytokines TNF-α and interferon-γ (Thurston et al. 2010) suggesting that KL plays an immunoregulatory role. Based on this knowledge, we assessed the role of KL in MOG-induced EAE. However, KL overexpression in the KL-OE mice had no effect on the development of the disease, as was reflected from the clinical scores and weight assessment. These results could be explained by the different role and distribution of KL in the brain and spinal cord as well as by the potential differential responses of OPCs to KL in CC and spinal cord. The secreted form of KL is detectable in plasma and cerebrospinal fluid (CSF) (Imura et al. 2004), and KL-KO mice show atrophy and dysfunction of the spinal anterior horn cells, while the posterior horn is well preserved (Anamizu et al. 2005), suggesting that KL plays a distinct role in various regions of the spinal cord. Moreover, we demonstrated that KL is expressed in the mouse CC, optic nerve, and spinal cord, with the highest expression in the CC and lowest in spinal cord (Chen et al. 2013). Higher KL levels in CC and lower levels in spinal cord imply that KL needs and functions may differ between these regions of the CNS and could explain the different effects of KL overexpression in the cuprizone and EAE models. For example, the conditional deletion of the transcription factor Ascl1 (Mash1) post-neurogenesis shows that Ascl1 is required during oligodendrogenesis for generating the correct numbers of white matter, but not gray matter OPCs, suggesting that these cells are not created equally (Vue et al. 2014). Also, mTOR was shown to be necessary for proper OL differentiation and myelination in the spinal cord, but not in the cortex. These data demonstrate that the requirement for mTOR varies by region with the spinal cord most dependent on mTOR (Wahl

et al. 2014). Moreover, a recent report reveals that the brain and spinal cord exhibit distinct sensitivities to cellular mediators of tissue damage and that IL-17 and IFN-γ differentially regulate recruitment of these mediators to each microenvironment (Simmons et al. 2014). Thus, differences in the requirement for KL between OLs in the brain and spinal cord could explain the lack of effect of KL overexpression in the EAE model.

In the cuprizone model, rapamycin was given to mice during the period of demyelination to inhibit OPC differentiation and spontaneous myelin repair and discontinued at the end of 6 weeks to allow for remyelination for 3 weeks. Interestingly, the only time when KL overexpression exhibited a beneficial effect compared to wild-type mice was during the remyelination period in the absence of rapamycin. Rapamycin is an inhibitor of mTORC1 (Cardenas et al. 1995) and indirectly and cell specifically of mTORC2 (Sarbassov et al. 2006). We have shown previously that KL promotes maturation of OPCs into myelin-producing OLs via ERK and Akt signaling (Chen et al. 2013), possibly leading to mTOR activation. mTOR activation has not been tested in the KL-induced OPC maturation system. The lack of the effect of KL during demyelination could be explained by the fact that the mTOR pathway is already inhibited by rapamycin, thus it cannot be affected by KL. Further studies are required to understand the KL/mTORC1 or KL/mTORC2 dialog in de/remyelination.

The recruitment of OPCs into areas of demyelination and their subsequent differentiation into mature, myelinating OLs is essential for CNS remyelination (Keirstead and Blakemore 1997; Trapp et al. 1997; Chang et al. 2002). White matter chronic demyelinated lesions in the brain of MS patients are characterized by the presence of OPCs (Chang et al. 2000) with impaired ability to differentiate into myelin-producing cells (Chang et al. 2000; Wolswijk 2000; Chang et al. 2002; Franklin 2002; Kuhlmann et al. 2008), and the lesions with extensive death of OLs are characterized by a repopulation of OPCs that are depleted over time (Levine and Reynolds 1999; Sim et al. 2002). Chronically demyelinated lesions are characterized by the presence of severely injured axons (Barnes et al. 1991; Trapp et al. 1998), but these demyelinated axons can be remyelinated under conditions of acute inflammation (O'Leary et al. 2002; Miron et al. 2011). In our previous study, we have shown that KL did not affect OPC cell number in vitro, but significantly increased the percentage of the mature OLs (Chen et al. 2013). Thus, we suggest that KL's enhanced remyelination in the corpus callosum of KL-OE mice following cuprizone-induced demyelination is due to its effect on OL differentiation and maturation (Chen et al. 2013) and that the presence of increased endogenous KL likely modifies the environment toward a permissive environment for remyelination. As an anti-aging protein, KL is not only associated with MS remyelination deficiency but also with the age-associated decrease in remyelination efficiency attributed to an impairment of OPC recruitment and the subsequent differentiation of the OPCs into myelinating oligodendrocytes (Sim et al. 2002; Chari et al. 2003). These findings support further the role of KL in ameliorating the MS- and age-associated decline in remyelination efficiency through its effect on OPCs maturation.

Surprisingly, KL-OE mice showed no significant difference in the amount of PDGFRα or GSTpi-positive cells after 3 weeks of spontaneous remyelination following 6 weeks of demyelination within the CC. These data suggest that the same number of mature OLs within the KL-OE CC compared to their WT littermates exist at the end of remyelination. We thus propose that individual OLs in the KL-OE mice have the potential of sending more processes to myelinate more axons during the remyelination phase as evidenced by the almost doubling of the number of myelinated axons in the CC after spontaneous remyelination. Further studies are required for testing this hypothesis.

Remarkably, we have found KL overexpression almost doubled the extent of remyelination in the corpus callosum, a white matter rich structure, but not in the hippocampus, a gray matter rich region. The generation of new OPCs and myelin can be observed in white matter lesions at the early stages of the disease (Prineas et al. 1993; Lucchinetti et al. 1999; Chang et al. 2012), while the majority of chronically demyelinated lesions in white matter demonstrate restricted remyelination due to a reduced recruitment of OPCs, the failure of OPCs to produce mature oligodendrocytes, and the insufficient production of myelin sheath by OLs (Trapp et al. 1997; Wolswijk 2000; Chang et al. 2002; Kuhlmann et al. 2008; Chang et al. 2012). Importantly, it was also shown that in the corpus callosum, premyelinating OLs usually occur in groups and their processes overlap extensively, which allows the clusters of OLs to compete more effectively for the survival factors in the neighborhood (Trapp et al. 1997). In postmortem brains of MS patients, cortical demyelination might outpace the demyelination of the white matter (Bo et al. 2003), but the benefits of active remyelination of cortical lesions, accompanied by a greater number of remyelinating OLs and fewer reactive astrocytes, have been demonstrated (Chang et al. 2012).

KL-KO mice demonstrate learning and memory deficits (Nagai et al. 2003), a reduced number of hippocampal synapses (Li et al. 2004), and changes associated with neurodegeneration in the hippocampus (Shiozaki et al. 2008). Interestingly, humans homozygous for a polymorphism in the KL gene, KL-VS, display diminished cognitive performance (Nagai et al. 2003; Deary et al. 2005), as well as reduced life span (Nagai et al. 2000; Arking et al. 2003; Arking et al. 2005). On the contrary, we demonstrated that human carriers heterozygous for the KL-VS variant have higher levels of KL in their blood and present with enhanced

cognition compared to individuals not carrying this polymorphism. The same is true with KL-OE mice that perform better on a number of behavioral tasks (Dubal et al. 2014). Moreover, we described biochemical differences between wild-type KL (KL-WT) and KL-VS in the form of a better binding to the FGF1 receptor of the KL-VS protein (Tucker Zhou et al. 2013). The roles KL plays in the brain, despite its clear importance for brain function based on the effects in KL-KO mice and of human polymorphisms, are not known, and a more thorough assessment of KL's molecular mechanisms of action is needed.

We have recently reported that KL is neuroprotective to primary rat and mouse neurons via a redox mechanism (Zeldich et al. 2014). Thus, KL may play a doubly beneficial role in demyelination: as an inducer of remyelination and as a neuroprotective factor for the damaged axons.

In conclusion, this is the first in vivo description for the role of KL as a regulator of remyelination in the white matter and as a new potential therapeutic target for myelin repair in MS. Finally, we conducted a high throughput screen and identified small molecules compounds that enhance KL expression. These molecules could become novel therapeutics for MS and other demyelinating disorders (Abraham et al. 2012; King et al. 2012b).

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