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Effects of the CRMP2 activator lanthionine ketimine ethyl ester on oligodendrocyte progenitor cells

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ABSTRACT

We previously showed LKE (lanthionine ketimine ester) reduces disease in the EAE model of multiple sclerosis, however whether LKE affects oligodendrocytes (OLGs) was not tested. In OLG progenitor cells (OPCs), LKE increased process number and area, but not PDGF-receptor-alpha expressing cells. In contrast, PDGF increased OPC numbers, but reduced process number and area. LKE increased collapsin response mediator protein-2 (CRMP2) expression, an LKE target, and CRMP2-expressing OLGs expressed myelin basic protein. LKE increased markers of OPC maturation, while PDGF, but not LKE, increased Sox2 expression. Our findings suggest that effects on OPCs may contribute to LKE beneficial actions in EAE.

1. Introduction

LKE (lanthionine ketimine ethyl ester) is a derivative of lanthionine ketimine, a cyclized metabolite of amino acid lanthionine (Hensley et al., 2010a). In vitro, LKE exhibits neuroprotective and neuritogenic effects (Kotaka et al., 2017; Marangoni et al., 2018), and reduces inflammatory activation of microglial cells (Hensley et al., 2010a; Kotaka et al., 2017). It has been shown that LKE increases axonal grown in serotonergic, GABAergic, cholinergic and cerebellar granule neurons (Hubbard et al., 2013; Kotaka et al., 2017; Marangoni et al., 2018). In vivo, LKE provides benefit in mouse models of neurological diseases and disorders including ischemia, AD, and spinal cord injury (Hensley et al., 2013; Kotaka et al., 2017; Nada et al., 2012). We showed that LKE also reduces disease severity and neurodegeneration in the EAE (experimental autoimmune encephalomyelitis) model of multiple sclerosis (MS) (Dupree et al., 2015). In those studies, LKE reduced some forms of myelin damage (less incidence of axons with redundant myelin sheets), and significantly increased myelin thickness in the spinal cord and optic nerve. However, whether those actions of LKE on myelin were secondary to neuroprotective effects, or involved direct actions on oligodendrocytes (OLGs) was not determined.

The mechanism by which LKE exerts its neuroprotective effects is not yet fully established. Several proteins show strong binding affinity to LKE, namely collapsin response mediator protein-2 (CRMP2), syntaxin-binding protein 1 (STXBP1), and lanthionine synthetase component like protein 1 (LanCl1) (Hensley et al., 2010a). Of these, interactions of LKE with CRMP2 have been the most well characterized. CRMP2 is one of 5 members of the CRMP family of proteins (Charrier et al., 2003). CRMP2 is expressed in neurons throughout development in the CNS, where it has roles in axonal growth and neurite extension by facilitating deposition of tubulin monomers to the growing end of neurofilaments (Niwa et al., 2017). The neuritogenic activity of CRMP2 is regulated phosphorylation of residues at the C-terminal of the molecule. In particular, phosphorylation at serine residue 522 by the Cdk5 kinase leads to hyperphosphorylation, CRMP2 inactivation, and interruption of tubulin polymerization (Uchida et al., 2005). The ability of LKE to induce neurite outgrowth likely involves inhibition of Cdk5 kinase and maintenance of active CRMP2 (Hensley et al., 2011).

In addition to neurons, CRMP2 is also expressed in OPCs and differentiated OLGs in vivo (Kamata et al., 1998; Ricard et al., 2000). A functional role for CRMP2 in OLGs comes from studies which examine process extension and retraction (Ricard et al., 2000; Spassky et al., 2002) during exposure to lysophospholipids (Dawson et al., 2003), or oxidative stress (Fernandez-Gamba et al., 2012) which cause activation of Rho kinase, CRMP2 phosphorylation, and induction of retraction. PDGF has also been shown to regulate CRMP2 dephosphorylation at

Abbreviations: CNP, cyclic-nucleotide phosphodiesterase; CRMP2, collapsin response mediator protein 2; LKE, Lanthionine ketimine ethyl ester; OLG, oligodendrocyte; OPC, oligodendrocyte progenitor cell; PDGFRa, platelet derived growth factor receptor alpha

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threonine 514, involving activation of protein phosphatases 1 and 2B (PP1 and PP2B) (Sarhan et al., 2017), suggesting that CRMP2 is involved in PDGF-directed OPC migration. It has also been suggested that CRMP2 is involved in the polymerization of tubulin in OLGs as well as OPCs (Yoshimura et al., 2005). Together these findings suggest that modulation of CRMP2 may play a role in both LKE and PDGF effects on OPCs and OLGs.

In the present study, we describe the effect of LKE using primary cultures of rat OPCs. We characterized and quantified the changes due to LKE in expression of PDGFR-alpha (PDGFRa), a marker of OPCs and pre-myelinating OLGs; and changes in morphological features visualized by immunostaining for alpha-tubulin. As a control we compared results with LKE to those due to treatment with PDGF to determine if LKE influences post-mitotic OPCs. We addressed the question of whether LKE increases OPC differentiation by quantifying myelin basic protein (MBP) expression. In attempts to understand mechanisms of actions, we observed that PDGF, but not LKE, influenced the expression of transcription factor Sox2, which has been shown to be involved in OPC maturation and differentiation. The data suggest that LKE could play a role in remyelination during EAE, which may be due in part to direct effects on OLG numbers or maturation state.

2. Material and methods

2.1. Preparation of primary glial cells

Adult pregnant Sprague Dawley rats (Charles River Laboratories, Cambridge, MA) were housed in clear, polyethylene cages at 22 °C on a 12:12 light/dark cycle with food and water available ad-libitum. Mixed glial cultures were prepared from postnatal day 1 cortices using procedures described (Savchenko, 2013). In brief, following removal of meninges, dissected cortices were plated into flasks in Dulbecco's Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma) and antibiotics. Media were changed once per week. Primary oligodendrocyte progenitor cells (OPCs) were prepared from mixed glial cultures as described (McCarthy and de Vellis, 1980). After 14 days in vitro (DIV), flasks were shaken at 300 rpm for 25 min to remove microglia, then shaken at 250 rpm at 37 °C for 12 h to dislodge OPCs. The OPCs were collected by gentle centrifugation, then plated onto coverslips coated with ornithine and poly-D-lysine (PDL) and maintained in OPC medium (DMEM supplemented with 1% B27, 1% N2 supplement, 30 μM triiodothyronine (T3) and 50 μg/ml gentamycin). One day after plating, the cells were treated with LKE (20 μM, a dose we previously showed significantly increased process number and length in primary mouse cerebellar granule cells (Marangoni et al., 2018)) with or without PDGF (10 nM), or PDGF alone, and the medium was changed after 3 days with fresh LKE and PDGF. Both LKE and PDGF were dissolved in OPC media. Cells were fixed for immunostaining after 6 days in vitro. Controls were kept under the same conditions with no additions. Experiments were carried out on at least 3 different preparations of primary OPCs, obtained from different preparations of mixed glial cells.

2.2. Immunofluorescent staining

Following indicated treatments, cells were rinsed in PBS and fixed in 3% formaldehyde for 30 min, then rinsed in PBS and incubated in 3% BSA in the presence of 0.2% Nonidet detergent for 1 h. Cells were then incubated with primary antibodies in 1% BSA and 0.2% nonidet at 4 °C for 12 h, rinsed in PBS and incubated with secondary antibodies in 1% BSA for 1 h. DAPI was used for nuclei identification. Immunofluorescent staining was visualized using a Zeiss Axiophot 2 epifluorescent microscope (Zeiss, Oberkochen, Germany), and Axio Vision Software (version 4.7) used for image acquisition. The primary antibodies were rabbit polyclonal anti-CRMP2 antibody (1:1000) (AbCAM, Cat # 129082); goat anti-Sox2 (1:500) (Santa Cruz, Cat # Sc-17,320); rabbit anti-PDGFR-alpha (1:500) (Santa Cruz Cat # Sc-338); rat monoclonal anti-alpha-tubulin (1:1000) (Millipore, Cat # MAB1864); and mouse monoclonal anti-MBP (1:100) (Millipore Cat # MAB387). The secondary antibodies used were rhodamine-conjugated donkey anti-rabbit (1:800), and FITC-conjugated donkey anti-goat, anti-mouse, anti-chicken, and anti-rat (1:500) (Jackson ImmunoResearch Laboratories; West Grove, PA, USA).

2.3. Image analysis

Quantitative analysis of images was done using ImageJ software (National Institute of Health, Bethesda, MD, USA). OPCs and OLGs numbers, and the number of processes were quantified in at least 6 different images per condition. Process length was measured using freehand lines placed along the process extending from cell body to most distal point; total area circumscribed by processes was determined by extending lines from one process distal tip to the next and then calculating total area encompassed, from 8-bit Axiovision ZVI files. Cell counting was based on morphology and MBP staining (OPCs were MBP negative and had fewer number of processes; OLGs were MBP positive and has numerous processes). Selected lengths and areas were measured in pixels where 1 pixel is 0.256 μm in length, and area of 0.06 μm^2.

2.4. Quantitative real-time PCR

Total RNA was isolated from cells using Direct-zol RNA MicroPrep (Zymo Research, Irvine, CA, USA) according to instructions, then 1 μg converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems cat #4368814, ThermoFisher, Waltham, MA, USA). The cDNA was amplified using FastStart Universal SYBR Green Master mix (Applied Biosystems, cat #04913914001, Foster City, CA, USA) in a Corbett RotoGene real-time PCR machine (Qiagen, Germantown, MD). The relative levels of mRNA were calculated from threshold take-off cycle number and normalized to values measured for β-actin in same samples. Primers used are shown in Table 1.

2.5. Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Statistical comparisons between groups was performed using 1-way analysis of variance (ANOVA) followed by Tukey multiple

Table 1
Primers used for mRNA analysis.

Gene	Direction	Sequence
MBP	Fow	5'-GCATCCTTGACTCCATCGG-3'
	Rev	5'-AGTCCTTGTACATGTGGCAC-3'
NG2	Fow	5'-CAATGGACGGATAGCCTTGAG-3'
	Rev	5'-GAGAGGTCGAAGTGGAAGC-3'
CNP	Fow	5'-CTACTTTGGCAAGAGACCTCC-3'
	Rev	5'-AGAGATGGACAGTTTGAAGGC-3'
PDGFRa	Fow	5'-AAAGAGAAAGAGCTGGGAACC-3'
	Rev	5'-AAACGGTGGGAATGAAGGAG-3'
CRMP2	Fow	5'-TGGTTTCAGCTTGTCTGGTG-3'
	Rev	5'-TGACAGGAAGGTGCTGACTG-3'
Nestin	Fow	5'-CCTCAAGATGTCCCTTAGTCTG-3'
	Rev	5'-TCCAGAAAGCCAAGAGAAGC-3'
Nanog	Fow	5'-CGTGTTGGCTGCATTTGTCTG-3'
	Rev	5'-ATAGTGCACTGTCTCCGAAGC-3'
Oct4	Fow	5'-GGAGAGGGATGTGGTTCGAG-3'
	Rev	5'-CGGCCTCATACTCTTCTCGTT-3'
Sox2	Fow	5'-GAACTAGACTCCGGGCGATG-3'
	Rev	5'-CCCAGCAAGAACCCTTTCCT-3'
b-act	Fow	5'-CCTGAAGTACCCCATTGAACA-3'
	Rev	5'-CACACGCAGCTCATTGTAGAA-3'
a-tub	Fow	5'-CCCTCGCCATGGTAAATACAT-3'
	Rev	5'-ACTGGATGGTACGCTTGGTCT-3'

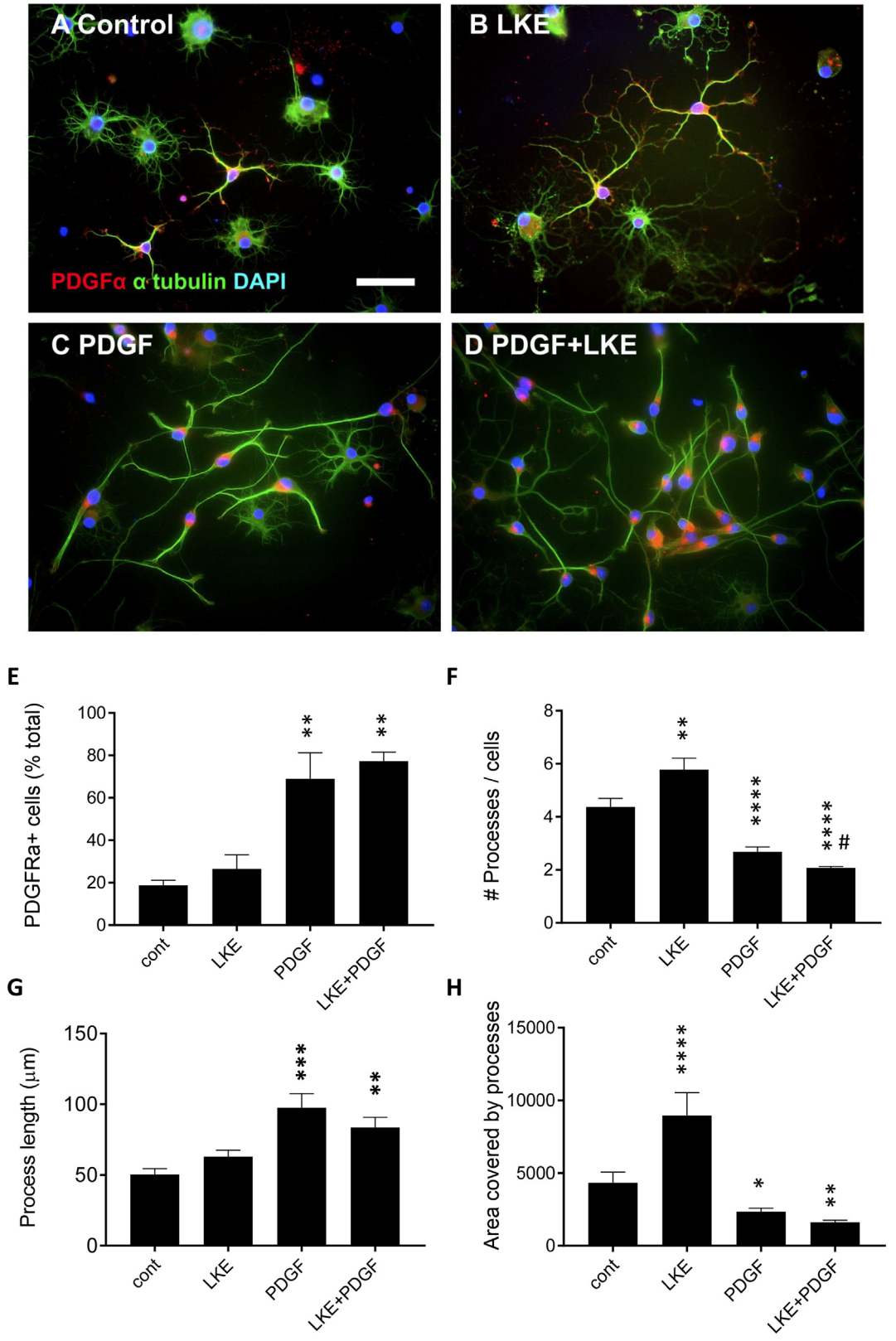


Fig. 1. Effects of LKE and PDGF on OPC maturation.
Immunofluorescent staining of OPCs for PDGFRa (red) after 6 days of treatment with (A) control media; (B) LKE; (C) PDGF; and (D) PDGF and LKE. The morphological features of the cells were visualized by co-staining for alpha-tubulin (green) and nuclei were labeled with DAPI (blue). Scale bar is 50 uM. The OPCs were imaged and quantified for (E) percentage of PDGFRa+ cells; average of 90 cells counted per condition; (F) processes per cell; average of 16 cells imaged per condition; (G) length of alpha-tubulin stained processes; average of 25 processes measured per condition; (H) area of alpha-tubulin stained branches average of 21 branches measured per condition. Data is mean ± SEM, *, $p < .05$; **, $p < .01$; ***, $p < .001$; **** $p < .0001$ versus control; #, $p < .05$ versus PDGF alone (1-way ANOVA, Tukey's multiple comparison test).

comparisons. Significance was set at $p < .05$. Calculations were carried out using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA) and Microsoft Excel 2016 Software.

3. Results

3.1. Effects of LKE and PDGF on OPC numbers and morphology

The effects of LKE on OPC numbers and morphology was examined in primary rat OPCs and compared to that due to the mitogen PDGF. PDGFRα, a marker of OPCs and non-myelinating OLGs, was observed in control cells in both cell bodies and branched processes, often seen near growth cones (Fig. 1A). Under control conditions, there were a few differentiated OLGs identified by the presence of a complex network of multiple branched processes, large cell body, and minimal PDGFRa+ expression. In the presence of 20 μM LKE, PDGFRα immunoreactive (IR) OPCs contained longer processes and PDGFRα expression was evident along the entire process length (Fig. 1B). Incubation of OPCs with PDGF decreased expression of PDGFRα, and the cells contained longer processes and appeared less branched (Fig. 1C). The reduction of PDGFRa+ staining was evident along alpha-tubulin labeled processes; and it was expressed mainly in the cytosol after PDGF treatment. PDGF increased the number of PDGFRα-IR OPCs, which was further increased when LKE was also present (Fig. 1D).

Quantitative analysis confirmed that the percentage of PDGFRα-IR cells was significantly increased approximately 4-fold by PDGF or PDGF plus LKE (Fig. 1E). However, LKE alone did not significantly alter the percentage of PDGFRα -IR cells. Quantitative analysis of the number and the length of OPC processes was done based on co-localization of α-tubulin with PDGFRα. The number of processes per cell was increased by LKE, decreased by PDGF, and further decreased by PDGF plus LKE (Fig. 1F). Process length in PDGFRα-IR OPCs was increased approximately 2-fold by PDGF, and to a slightly smaller extent (about 1.7-fold) by PDGF plus LKE. Although some processes were extremely long following LKE treatment, the average number of processes was not significantly increased as compared to controls (Fig. 1G). The average total area encompassed by cell process branches (Fig. 1H) was increased about 2-fold by LKE and decreased by PDGF or by PDGF plus LKE. These findings are consistent with a proliferative effect of PDGF (increased number of OPCs, fewer processes per cell, reduced total branched area) and suggest a differentiating action of LKE (increased number of processes per cell and increased total branched area).

3.2. Effects of LKE and PDGF on CRMP2 expression

In control OPCs, CRMP2 was detected in the majority of cells, with strong staining observed in OLGs with bushy branches, large cell bodies, and myelin sheaths; and at lower levels in OPCs with smaller cell bodies and fewer processes (Fig. 2A). LKE caused an increase of CRMP2 expression and the cells had longer processes compared to control cells (Fig. 2B). PDGF slightly reduced CRMP2 expression (Fig. 2C), while treatment with both led to CRMP2 expression in most cells (Fig. 2D). Quantitative analysis revealed that the percentage of CRMP2-IR cells was only slightly modified by LKE or by PDGF alone, but significantly increased by LKE together with PDGF (Fig. 2E).

To further test if LKE increased OLG differentiation, we examined expression of transcription factor Sox2, necessary for proliferation, maturation and differentiation (Zhang et al., 2018b) and priming for eventual differentiation and remyelination (Zhao et al., 2015). However, LKE did not alter the percentage of Sox2-ir cells, although treatment with PDGF alone, or PDGF+LKE increased the percentage of Sox2-ir cells by about 20% (Fig. 2F). Interestingly, there was a linear correlation between the number of CRMP2 and Sox2 labeled cells (Fig. 2G), suggesting that effects of PDGF on Sox2 may mediate the regulation of transcription in CRMP2 expressing cells.

CRMP2-IR OLGs also expressed MBP whereas smaller, non-branched OPCs did not express MBP (Fig. 3). LKE, alone or with PDGF increased MBP expression, which was evident in cell bodies, surrounding myelin sheath, and in growth cones as puncta. Treatment with PDGF (alone or with LKE) also increased MBP staining, but greatly increased the number of CRMP2-IR OPCs which were MBP negative. Quantitative analysis showed that the percentage of MBP-IR OLGs of the total number of CRMP2-IR cells was increased by LKE and decreased by PDGF or PDGF plus LKE (Fig. 3E). However, the percentage of MBP+: CRMP2+ cells was significantly increased by LKE and decreased by PDGF or PDGF+LKE (Fig. 3F).

CRMP2-IR expression in MBP-IR cells (both OPCs and OLGs) was increased by LKE, and significantly decreased by PDGF, with little change due to PDGF+LKE (Fig. 3G). Quantitative analysis of the CRMP2-IR OPCs and OLGs (Fig. 3H) was done based on morphological features and the expression of MBP to define mature OLGs. While LKE did not alter the percentage of CRMP2-IR OPCs or OLGs, treatment with PDGF or PDGF+LKE increased CRMP2-IR OPCs, with a corresponding decrease in the percentage of CRMP2-IR OLGs.

3.3. Effects of LKE on OPC gene expression

To determine if LKE affected OPC or OLG gene expression, we measured levels of early and late markers of differentiation (Fig. 4). At 24 h after treatment, we observed modest but non-significant reductions of early stem cell markers Nanog and Oct4, and a significant increase in the early OPC marker nestin mRNA. LKE significantly increased PDGFRa but did not alter NG2 mRNA levels. LKE caused a small but non-significant increase in CNPase, and a significant increase in MBP. The increase in CRMP2 mRNA almost reached statistical significance ($P = .067$).

4. Discussion

The current findings demonstrate that LKE induces significant changes in OPC process number and area, without an increase the number of OPCs. In contrast, incubation with PDGF significantly increases the number of OPCs, consistent with its activity as a mitogen. The increased number of new OPCs likely accounts for the reduction in the average number of processes per cell, and the total branched area of the processes. The observation that PDGF, but not LKE increased process length may be due to an increased number of immature OPCs that are bipolar, while an increase in total area by LKE could be due to process thickening. The mechanisms responsible for increased process length are not known; but may be similar to the effects of LKE on neuritogenesis which involves stabilization and elongation of neurofilaments (Marangoni et al., 2018). The low levels of PDGFRa expression in cells with a mature morphology (2 can be seen in Fig. 1A), together with the absence of mature appearing OLGs in the presence of PDGF (Fig. 1C) are consistent with a proliferative effect of PDGF. The differentiating action of LKE, suggested by the increase in the number of processes per cell and overall branch area, was not observed when cells were treated with LKE and PDGF (observed in Fig. 1B but not in Fig. 1C), suggesting that the proliferative action of PDGF overwhelms any differentiating actions of LKE. The effects of LKE on branching and differentiation are similar to what has been reported to occur in neurons (Marangoni et al., 2018), suggesting similar role for LKE in neurons and OPCs.

Our data are similar to those reported by (Baas et al., 2016; Rao and Baas, 2018) showing that neuronal microtubules are more stable in dividing cells, suggesting by results that CRMP2 expression is slightly increased by LKE, but decreased by PDGF (Fig. 3G). This suggests that microtubules in immature OPCs have a non-uniform polarity as observed in neuronal dendrites, whereas LKE increased microtubule interactions between CRMP2 and binding proteins such as b-tubulin, which stabilizes them, and may contribute to microtubule directionality. Changes in microtubule directionality in OLGs (e.g. uniformly

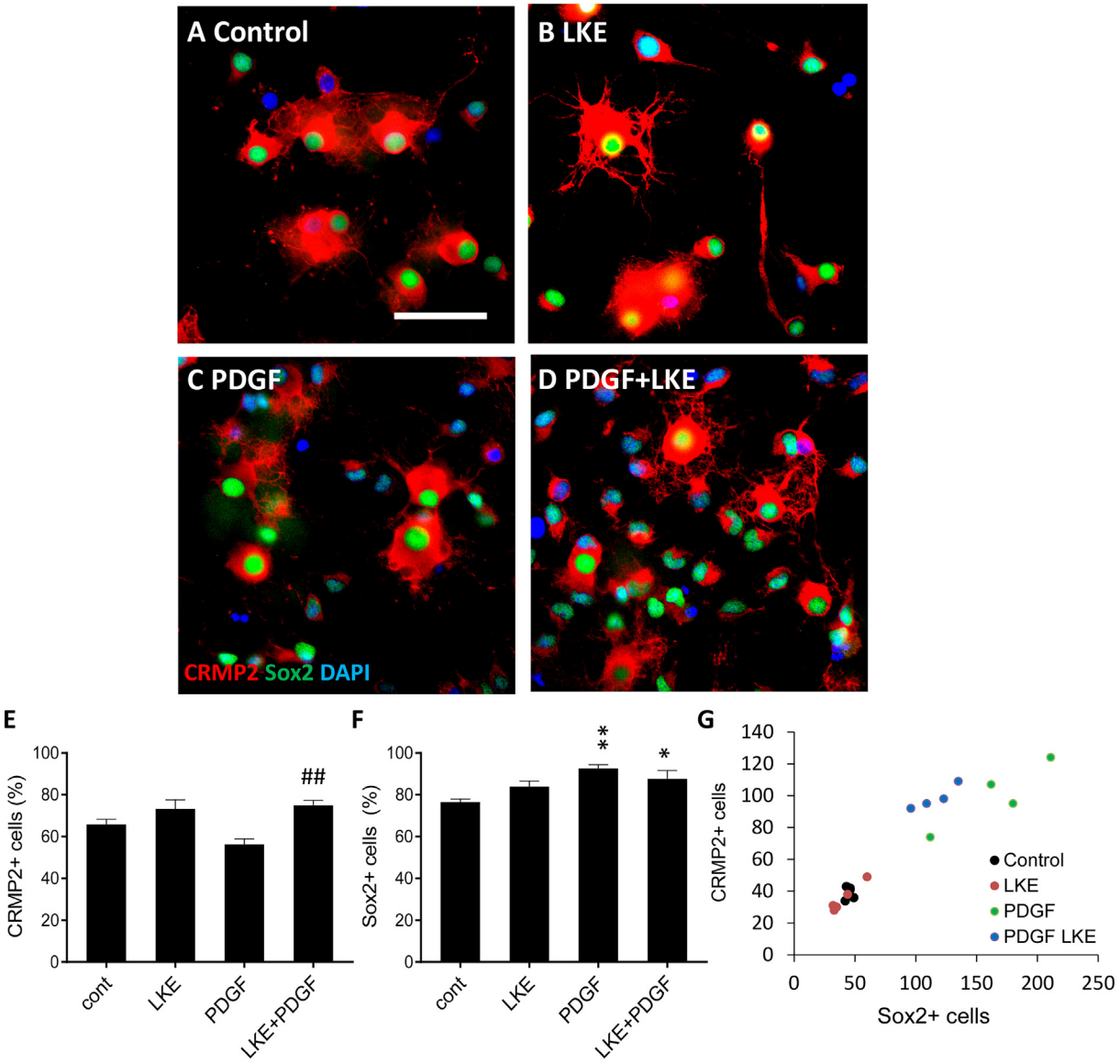
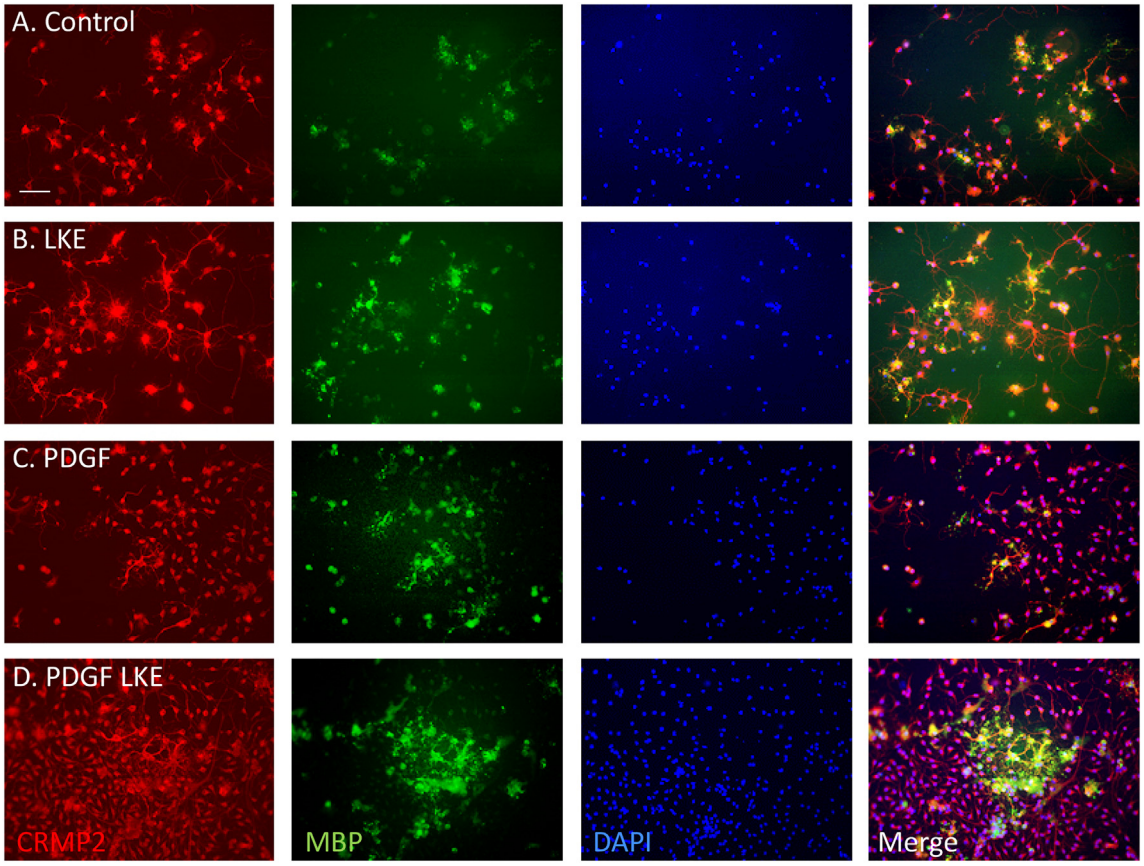


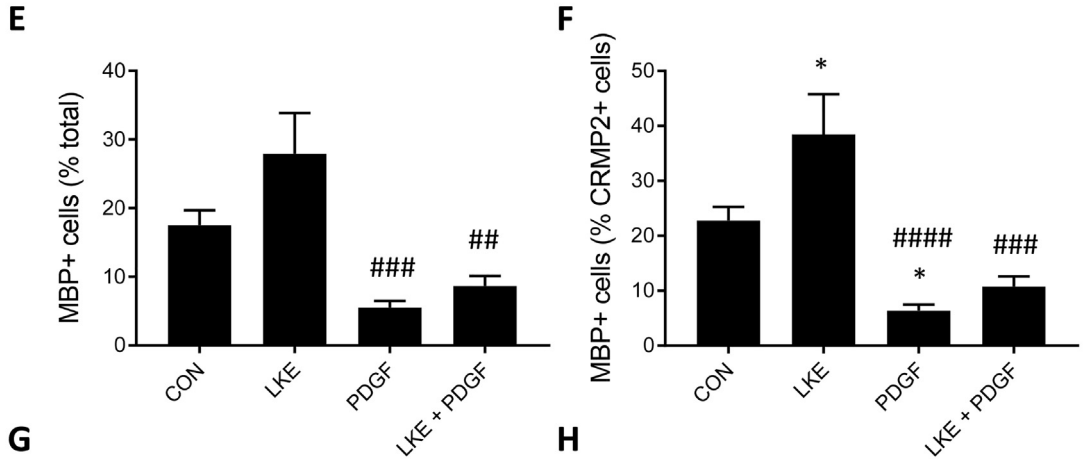
Fig. 2. Effects of LKE and PDGF on CRMP2 and Sox2 expression.
Immunofluorescent staining of OPCs for CRMP2 (red) and Sox2 (green) after 6 days treatment with (A) control media; (B) LKE; (C) PDGF; and (D) PDGF and LKE. Nuclei were labeled with DAPI (blue). Scale bar, 20 μm. The cells were imaged and quantified for the (E) percentage of CRMP2 + cells; (F) percentage of Sox2 + cells; (G) relative # of CRMP2: Sox2 positive cells. Data is mean ± SEM (average number of cells counted was 400 per condition). *, $p < .05$; **, $p < .01$ versus control; ##, $p < .01$ versus PDGF alone (1-way ANOVA, Tukey's multiple comparison test).

oriented in small processes, compared to a non-uniform distribution in large processes) may reflect the distinct functions of the different OLG regions, for example the presence of compact myelin required for normal axonal function (Kirkpatrick et al., 2001; Lunn et al., 1997).

Our findings that CRMP2 is expressed in both OPCs and mature OLGs is consistent with previous studies showing CRMP2 expression in these cells (Bretin et al., 2005; Ricard et al., 2000). In the current study, identified CRMP2 by immunostaining in both immature (uni and bipolar, MBP negative OPCs) as well as in mature MBP stained OLGs. Previous RNAseq studies (Zhang et al., 2014) show that CRMP2 is expressed in OPCs, immature OLGs, and mature OLGs, with the highest expression being in the immature (non-myelinating OLGs). This also explains why we could detect CRMP2 under control conditions. Since CRMP2 is one of a member of 5 related proteins (Moutal et al., 2019), it is also likely that other CRMP2 isoforms are expressed differentially at different time points during OLG maturation, some of which are also phosphorylated by Cdk5 kinase and therefore also regulated by LKE.

Our findings that LKE leads to increases in process numbers and area are in line with studies using semaphorins to activate CRMP2. Class 3 semaphorins (Sema3s) bind to receptors comprised of plexins and neuropilins, both which are expressed on OPCs and OLGs (Piaton et al., 2011; Syed et al., 2017) and which upon binding of class 3 semaphorins (Sema3s) can activate CRMP2 (as well as other signaling pathways). Findings that incubation of mature OLGs with Sema3A causes collapse of processes first suggested a role for CRMP2 (Ricard et al., 2000). CRMP2 activation also effects migration, as indicated by studies using optic nerve explants. When placed in culture, OPCs migrate out from embryonic day 16 optic nerve explants; and that migration is repulsed by Sema3A and attracted by Sema3F (Spassky et al., 2002). Similarly, in the adult mouse brain OPCs migration towards lesion sites was increased by introduction of Sema3F, and decreased by Sema3A (Piaton et al., 2011). Activation of CRMP2 by other methods also effects OPC processes. Treatment of primary rat OPCs to a non-lethal oxidative stress induced by low doses of nitro-proprionic acid led to process retraction that was correlated to increased CRMP2 phosphorylation (Fernandez-Gamba et al., 2012). Together, these studies





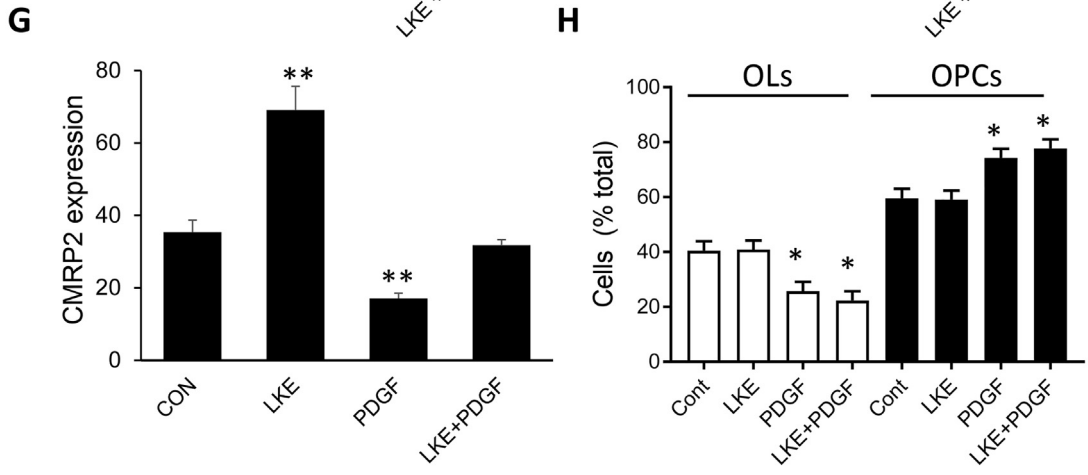


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Fig. 3. MBP expression of MBP in CRMP2+ OLGs.
Immunofluorescent staining of OPCs for CRMP2 (red) and MBP (green) after 6 days treatment with (A) control media; (B) LKE; (C) PDGF; and (D) PDGF and LKE. Nuclei labeled with DAPI (blue). Scale bar is 80 μM. Quantitation for (E) the percentage of MBP+ OLGs of total cells; (F) the percentage of MBP+ OLGs of the CRMP2+ cells and (G) CRMP2 expression in OLGs. (H) shows the percentage of CRMP2+ cells that were either OLGs or OPCs based on morphology. Data is mean ± SEM, average number of cells was 300 cells per condition. *, $p < .05$ versus control; ##, $p < .05$, ###, $p < .005$; ####, $p < .0001$ versus LKE.

suggest that CRMP2 activation leads to process retraction as well as regulation of migration. Increases in OPC process number and area covered, without robust increases in maturation, could therefore be related to regulation of OPC polarity or migration.

Whether the effects of LKE on OPCs is due to regulation of CRMP2 and/or of other targets remains to be determined. Proteomic studies identified several proteins which bind tightly to LKE, including CRMP2, STXBP1 (syntaxin binding protein 1), and LanCL1 (lanthionine synthetase C-like protein 1) (Hensley et al., 2010a). STXBP1 is a key component of the synaptic vesicle release machinery (Rizo, 2018), while LanCL1 is involved in regulation of glutathione levels (Hensley et al., 2010b). While neither of these proteins have been reported to be expressed in OPCs or OLGs, we cannot rule out the possibility that some of LKE actions are also mediated via effects on one or both of these targets.

PDGF has been shown to induce the proliferation of OPCs and play a role in OLG differentiation and myelination (Betsholtz, 2004; Fruttiger et al., 1999); while deficiency of the PDGFRa impairs proliferation and regeneration of OLGs under pathological conditions (Murtie et al., 2005). Consistent with this, we find that PDGF increases the number of OPCs, as well as increasing the length of processes whereas reducing branched area and the number of processes per cell. The mechanism underlying PDGF effects are not fully known, but may involve activation of Shp2- pathway (Abbasi et al., 2018). Interestingly, a phosphoproteomics study has shown that PDGF leads to dephosphorylation of CRMP2 at Thr514 in a phosphatase 1 and 2B (PP1 and PP2B)-mediated pathway; and it was suggested that CRMP2 is required for PDGF-directed OPC migration in vitro (Sarhan et al., 2017). Such PDGF-induced dephosphorylation of CRMP2 could also account for the small, but significant further reduction in processes per cell due to treatment with PDGF & LKE (Fig. 1F); since changes in CRMP2 phosphorylation are known to alter is neurotrophic actions (Marangoni et al., 2018; Nada et al., 2012). Overall, the data suggests that the actions of LKE on OPCs may be mediated in part by reductions in CRMP2 phosphorylation; which is further regulated by PDGF.

We also observed that Sox2 expression in OPCs is regulated by PDGF, and its expression is correlated with the number of CRMP2+ cells (Fig. 2F,G). Sox2 is a member of a superfamily of proteins that contain the High Mobility Group (HMG) box DNA-binding domain and regulates stem cell self-renewal and neurogenesis (Chambers and Tomlinson, 2009). Sox2 is expressed in neuronal progenitor cells, early postnatal OPCs and in adult brain OPCs (Zhang et al., 2018b). Recent studies have established that Sox2 is expressed in OPCs and is essential for their proliferation and differentiation into OLGs (Kopp et al., 2008; Lee et al., 2013; Zhang et al., 2018b), and changes in its expression could induce OPCs to proliferate and differentiate under pathological conditions (Doi et al., 2017). Our results suggest that LKE increases OPC maturation, indicated by a greater number of processes, a greater percentage of cells that express MBP, and increases in mRNA levels of several factors involved in OLG differentiation (Nestin, PDGFRa, and MBP). However, we did not find any increase due to LKE in Sox2 expression at either the protein (Fig. 2F) or mRNA levels (Fig. 4), which is unexpected since Sox2 is involved in OLG differentiation. However, we found a strong correlation between the number of Sox2+ cells and CRMP2+ cells (Fig. 2G). This suggests that while there is a clear association between CRMP2 and Sox2 expression, that other factors which are induced by PDGF, but not LKE alone, work in concert with CRMP2 to induce maturation. Since Sox2 is also involved in regulating the timing of OPC differentiation (Zhang et al., 2018a), our results may be limited by the single time point that as analyzed. It is also the case that Sox2 has established roles in inducing OPC proliferation, which may lead to underestimation of Sox2 expression when the total cell population (OPCs and OLGs) is used for measurement (qRT-PCR or total immunolabeling). Nevertheless, the clear relationship between CRMP2+ and Sox2+ stained cells points to a role for this target of LKE in OLG maturation.

Our qRT-PCR results show that LKE alters gene expression patterns consistent with induction of OLG maturation. Following overnight incubation with LKE, we observed modest reductions of early stem cell markers Nanog and Oct4, and a significant increase in the early OPC marker nestin. We also observed higher levels of mature OLG markers (CNPase and to a greater extent MBP). These results are consistent with findings that in mice where CRMP2 phosphorylation at serine residue 522 is blocked (by replacing it with alanine residue), which is the same target of LKE, leads to increases in expression of several OLG genes (Nakamura et al., 2018). In several cases (PDGFRa, MBP, CRMP2) we found differences in the mRNA levels despite small, non-significant increase in the number of stained cells (Fig. 1E); this may be due to the differences in incubation times used for the different studies (1 day for qRT-PCR versus 6 days for immnostaining) or may reflect differences in mRNA translation or protein stability. Overall, while measurements of other factors are required, the results are consistent with the conclusion that LKE induces a shift to a more mature OLGs.

In summary, our data demonstrate that LKE, a known modulator of CRMP2 function by inhibiting Cdk5 kinase phosphorylation influences OPC morphology, and suggests LKE increases OPC differentiation towards OLGs. This may help account for the benefit observed in the EAE mouse model of MS, and suggests that LKE may have therapeutic potential in neurological disease and damage involving demyelination.

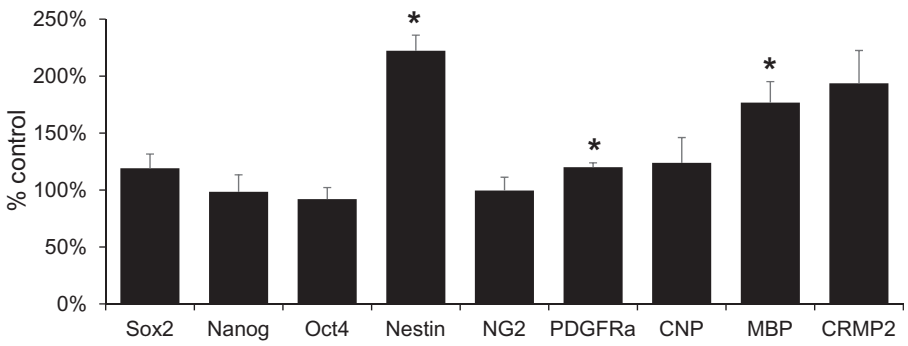

Fig. 4. Effects of LKE on OLG gene expression.
Primary OPCs were incubated with 20 μM LKE for 24 h, then levels of indicated mRNAs measured by qPCR, normalized to values for β-actin in the same samples. Data are mean ± sem, $n = 3$ per group and show relative expression in LKE-treated cells versus control cells. *, $p < .05$ versus control values.

Declaration of interest

None.

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MBP, myelin basic protein; NG2, chondroitin sulfate proteoglycan; CNP, cyclic nucleotide phosphodiesterase; PDGFRa, platelet derived growth factor receptor alpha; CRMP2, collapsing response mediator protein 2; Oct-4, octamer-binding transcription factor 4; Sox2, SRY-box 2; b-act, beta-actin.

References

Abbasi, M., Gupta, V., Chitranshi, N., You, Y., Dheer, Y., Mirzaei, M., et al., 2018. Regulation of brain-derived Neurotrophic factor and growth factor Signaling pathways by tyrosine phosphatase Shp2 in the retina: a brief review. Front. Cell. Neurosci. 12, 85.

Baas, P.W., Rao, A.N., Matamoros, A.J., Leo, L., 2016. Stability properties of neuronal microtubules. Cytoskeleton (Hoboken, NJ). 73, 442–460.

Betsholtz, C., 2004. Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev. 15, 215–228.

Bretin, S., Reibel, S., Charrier, E., Maus-Moatti, M., Auvergnon, N., Thevenoux, A., et al., 2005. Differential expression of CRMP1, CRMP2A, CRMP2B, and CRMP5 in axons or dendrites of distinct neurons in the mouse brain. J. Comp. Neurol. 486, 1–17.

Chambers, I., Tomlinson, S.R., 2009. The transcriptional foundation of pluripotency. Development (Cambridge, England) 136, 2311–2322.

Charrier, E., Reibel, S., Rogemond, V., Aguera, M., Thomasset, N., Honnorat, J., 2003. Collapsin response mediator proteins (CRMPs): involvement in nervous system development and adult neurodegenerative disorders. Mol. Neurobiol. 28, 51–64.

Dawson, J., Hotchin, N., Lax, S., Rumsby, M., 2003. Lysophosphatidic acid induces process retraction in CG-4 line oligodendrocytes and oligodendrocyte precursor cells but not in differentiated oligodendrocytes. J. Neurochem. 87, 947–957.

Doi, T., Ogata, T., Yamauchi, J., Sawada, Y., Tanaka, S., Nagao, M., 2017. Chd7 collaborates with Sox2 to regulate activation of Oligodendrocyte precursor cells after spinal cord injury. J. Neurosci. 37, 10290–10309.

Dupree, J.L., Polak, P.E., Hensley, K., Pelligrino, D., Feinstein, D.L., 2015. Lanthionine ketimine ester provides benefit in a mouse model of multiple sclerosis. J. Neurochem. 134, 302–314.

Fernandez-Gamba, A., Leal, M.C., Maarouf, C.L., Richter-Landsberg, C., Wu, T., Morelli, L., et al., 2012. Collapsin response mediator protein-2 phosphorylation promotes the reversible retraction of oligodendrocyte processes in response to non-lethal oxidative stress. J. Neurochem. 121, 985–995.

Fruttiger, M., Karlsson, L., Hall, A.C., Abramsson, A., Calver, A.R., Bostrom, H., et al., 1999. Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. Development (Cambridge, England) 126, 457–467.

Hensley, K., Christov, A., Kamat, S., Zhang, X.C., Jackson, K.W., Snow, S., et al., 2010a. Proteomic identification of binding partners for the brain metabolite lanthionine ketimine (LK) and documentation of LK effects on microglia and motoneuron cell cultures. J. Neurosci. 30, 2979–2988.

Hensley, K., Venkova, K., Christov, A., 2010b. Emerging biological importance of central nervous system lanthionines. Molecules (Basel, Switzerland) 15, 5581–5594.

Hensley, K., Venkova, K., Christov, A., Gunning, W., Park, J., 2011. Collapsin response mediator protein-2: an emerging pathologic feature and therapeutic target for neurodisease indications. Mol. Neurobiol. 43, 180–191.

Hensley, K., Gabbita, S.P., Venkova, K., Hristov, A., Johnson, M.F., Eslami, P., et al., 2013. A derivative of the brain metabolite lanthionine ketimine improves cognition and diminishes pathology in the 3 x Tg-AD mouse model of Alzheimer disease. J. Neuropathol. Exp. Neurol. 72, 955–969.

Hubbard, C., Benda, E., Hardin, T., Baxter, T., St John, E., O'Brien, S., et al., 2013. Lanthionine ketimine ethyl ester partially rescues neurodevelopmental defects in unc-33 (DPYSL2/CRMP2) mutants. J. Neurosci. Res. 91, 1183–1190.

Kamata, T., Subleski, M., Hara, Y., Yuhki, N., Kung, H., Copeland, N.G., et al., 1998. Isolation and characterization of a bovine neural specific protein (CRMP-2) cDNA homologous to unc-33, a C. elegans gene implicated in axonal outgrowth and guidance. Brain research. Mol. Brain Res. 54, 219–236.

Kirkpatrick, L.L., Witt, A.S., Payne, H.R., Shine, H.D., Brady, S.T., 2001. Changes in microtubule stability and density in myelin-deficient shiverer mouse CNS axons. J. Neurosci. 21, 2288–2297.

Kopp, J.L., Ormsbee, B.D., Desler, M., Rizzino, A., 2008. Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells (Dayton, Ohio) 26, 903–911.

Kotaka, K., Nagai, J., Hensley, K., Ohshima, T., 2017. Lanthionine ketimine ester promotes locomotor recovery after spinal cord injury by reducing neuroinflammation and promoting axon growth. Biochem. Biophys. Res. Commun. 483, 759–764.

Lee, H.J., Wu, J., Chung, J., Wrathall, J.R., 2013. SOX2 expression is upregulated in adult spinal cord after contusion injury in both oligodendrocyte lineage and ependymal cells. J. Neurosci. Res. 91, 196–210.

Lunn, K.F., Baas, P.W., Duncan, I.D., 1997. Microtubule organization and stability in the oligodendrocyte. J. Neurosci. 17, 4921–4932.

Marangoni, N., Kowal, K., Deliu, Z., Hensley, K., Feinstein, D.L., 2018. Neuroprotective and neurotrophic effects of Lanthionine Ketimine Ester. Neurosci. Lett. 664, 28–33.

McCarthy, K.D., de Vellis, J., 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J. Cell Biol. 85, 890–902.

Moutal, A., White, K.A., Chefdeville, A., Laufmann, R.N., Vitiello, P.F., Feinstein, D., et al., 2019 Mar 27. Dysregulation of CRMP2 post-translational modifications drive its pathological functions. Mol. Neurobiol. https://doi.org/10.1007/s12035-019-1568-4. [Epub ahead of print] Review. PMID: 30915713.

Murtie, J.C., Zhou, Y.X., Le, T.Q., Vana, A.C., Armstrong, R.C., 2005. PDGF and FGF2 pathways regulate distinct oligodendrocyte lineage responses in experimental demyelination with spontaneous remyelination. Neurobiol. Dis. 19, 171–182.

Nada, S.E., Tulsulkar, J., Raghavan, A., Hensley, K., Shah, Z.A., 2012. A derivative of the CRMP2 binding compound lanthionine ketimine provides neuroprotection in a mouse model of cerebral ischemia. Neurochem. Int. 61, 1357–1363.

Nakamura, H., Takahashi-Jitsuki, A., Makihara, H., Asano, T., Kimura, Y., Nakabayashi, J., et al., 2018. Proteome and behavioral alterations in phosphorylation-deficient mutant Collapsin response mediator Protein2 knock-in mice. Neurochem. Int. 119, 207–217.

Niwa, S., Nakamura, F., Tomabechi, Y., Aoki, M., Shigematsu, H., Matsumoto, T., et al., 2017. Structural basis for CRMP2-induced axonal microtubule formation. Sci. Rep. 7, 10681.

Piaton, G., Aigrot, M.S., Williams, A., Moyon, S., Tepavcevic, V., Moutkine, I., et al., 2011. Class 3 semaphorins influence oligodendrocyte precursor recruitment and remyelination in adult central nervous system. Brain 134, 1156–1167.

Rao, A.N., Baas, P.W., 2018. Polarity sorting of microtubules in the axon. Trends Neurosci. 41, 77–88.

Ricard, D., Stankoff, B., Bagnard, D., Aguera, M., Rogemond, V., Antoine, J.C., et al., 2000. Differential expression of collapsin response mediator proteins (CRMP/ULIP) in subsets of oligodendrocytes in the postnatal rodent brain. Mol. Cell. Neurosci. 16, 324–337.

Rizo, J., 2018. Mechanism of neurotransmitter release coming into focus. In: Protein Science. vol. 27. A publication of the Protein Society, pp. 1364–1391.

Sarhan, A.R., Szyroka, J., Begum, S., Tomlinson, M.G., Hotchin, N.A., Heath, J.K., et al., 2017. Quantitative Phosphoproteomics reveals a role for Collapsin response mediator protein 2 in PDGF-induced cell migration. Sci. Rep. 7, 3970.

Savchenko, V.L., 2013. Regulation of NADPH oxidase gene expression with PKA and cytokine IL-4 in neurons and microglia. Neurotox. Res. 23, 201–213.

Spassky, N., de Castro, F., Le Bras, B., Heydon, K., Queraud-LeSaux, F., Bloch-Gallego, E., et al., 2002. Directional guidance of oligodendroglial migration by class 3 semaphorins and netrin-1. J. Neurosci. 22, 5992–6004.

Syed, Y.A., Abdulla, S.A., Kotter, M.R., 2017. Studying the effects of Semaphorins on Oligodendrocyte lineage cells. Methods Mol. Biol. (Clifton, NJ) 1493, 363–378.

Uchida, Y., Ohshima, T., Sasaki, Y., Suzuki, H., Yanai, S., Yamashita, N., et al., 2005. Semaphorin3A Signalling Is Mediated Via Sequential Cdk5 and GSK3beta Phosphorylation of CRMP2: Implication of Common Phosphorylating Mechanism Underlying Axon Guidance and Alzheimer's Disease. Genes to Cells: Devoted to Molecular & Cellular Mechanisms. vol. 10. pp. 165–179.

Yoshimura, T., Kawano, Y., Arimura, N., Kawabata, S., Kikuchi, A., Kaibuchi, K., 2005. GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. Cell. 120, 137–149.

Zhang, Y., Chen, K., Sloan, S.A., Bennett, M.L., Scholze, A.R., O'Keeffe, S., et al., 2014. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J. Neurosci. 34, 11929–11947.

Zhang, S., Rasai, A., Wang, Y., Xu, J., Bannerman, P., Erol, D., et al., 2018a. The stem cell factor Sox2 is a positive timer of Oligodendrocyte development in the postnatal murine spinal cord. Mol. Neurobiol. 55, 9001–9015.

Zhang, S., Zhu, X., Gui, X., Croteau, C., Song, L., Xu, J., et al., 2018b. Sox2 is essential for Oligodendroglial proliferation and differentiation during postnatal brain myelination and CNS Remyelination. J. Neurosci. 38, 1802–1820.

Zhao, C., Ma, D., Zawadzka, M., Fancy, S.P., Elis-Williams, L., Bouvier, G., et al., 2015. Sox2 sustains recruitment of Oligodendrocyte progenitor cells following CNS demyelination and primes them for differentiation during Remyelination. J. Neurosci. 35, 11482–11499.