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Alterations in both caliber and myelination of ~~adult~~ callosal axons elicited by ubiquitous genetic ablation of c-Jun amino-terminal kinase 3 (JNK3).

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ABBREVIATIONS: Cldn11: claudin-11; CC: corpus callosum; JNK3: cJun amino-terminal kinase 3; MAPKs: mitogen-activated protein kinases; NFs: neurofilament; NFH: neurofilament heavy chain; NFL: neurofilament light chain; OPC: oligodendrocyte precursor cell; Plp1: myelin proteolipid protein; Mog: myelin-oligodendrocyte glycoprotein; TEM: transmission electron microscopy; WT: wild type.

ABSTRACT

Myelination is a fundamental process supporting appropriate motor, sensory, and cognitive functions. During development, oligodendrocyte progenitor cells (OPCs) proliferate, migrate, and gradually differentiate into mature oligodendrocytes, which produce and assemble myelin sheets that insulate axons in the mammalian central nervous system. Recent evidence suggested a regulatory role of the protein kinase JNK1, one of three mammalian JNK isoforms, on the proliferation and differentiation of OPCs, but whether other JNK isoforms modulate these and other cellular events contributing to myelination has not yet been explored. Building on results from an unbiased proteomic analysis, our studies here revealed increased numbers of OPCs, but not mature oligodendrocytes, in the corpus callosum of ~~adult~~ mice featuring germline ablation of the JNK3 isoform. Ultrastructural analyses further showed increased proportion of small caliber callosal axons in these mice, as well as thinning of their myelin sheaths. These alterations were accompanied by reduced phosphorylation of heavy chain subunits of neurofilaments (NFs), major cytoskeletal elements linking myelin to the regulation of axonal caliber. Collectively, our findings reveal previously unrecognized effects of JNK3 deletion on OPC proliferation, NF phosphorylation, callosal axon caliber and myelin thickness *in vivo*, suggesting a potential involvement of this kinase on myelinogenesis and/or myelin maintenance.

INTRODUCTION

Myelin, a lipid- and membrane protein-rich structure that wraps axons in a spiral fashion, allows high-speed conduction of action potentials, facilitating the synchronization of impulse discharges among distant neurons and functionality of neuronal circuits (Nishiyama *et al.* 2021). The initial assembly and subsequent thickening of a compact myelin sheath involve various interactions among proteins in both oligodendrocyte and axonal surfaces (Duncan *et al.* 2021; Piaton *et al.* 2010). Beyond their role in insulating axons, myelin sheaths also influence axonal caliber by locally modulating the phosphorylation of neurofilaments (NFs), major cytoskeletal components supporting axonal structure and caliber (Brady *et al.* 1999; Waegh *et al.* 1992; Kirkpatrick and Brady 1994). Such influence is best reflected in the nodes of Ranvier, axonal subdomains devoid of myelin that feature a marked reduction in caliber and a localized enrichment in dephosphorylated NFs (Hoffman 1995). The essential role of myelin in motor, sensory, and cognitive functions is highlighted by deleterious neurological phenotypes elicited by mutations in critical myelin components and by the marked loss of myelin observed in a wide variety of neurodegenerative disorders (Duncan and Radcliff 2016; Stadelmann *et al.* 2019; Bardile *et al.* 2023; Schäffner *et al.* 2023). An understanding of the mechanisms and specific molecular components contributing to the myelination process could provide a much-needed framework for the development of therapies based on preventing myelin dysfunction in these diseases.

During development, oligodendrocyte precursor cells (OPCs) proliferate and migrate to populate the entire central nervous system (CNS) (Bergles and Richardson 2015). After this, OPCs undergo a complex maturation process that culminates with their differentiation into oligodendrocytes, the cell type directly responsible for the production of the lipid and protein components of myelin, as well as their assembly into a functional myelin sheath (Bergles and Richardson 2015). In adulthood, OPCs retain their proliferative capacity and their ability to differentiate into oligodendrocytes to sustain myelination (Dawson *et al.* 2003; Young *et al.* 2013). OPC differentiation into mature oligodendrocytes involves major changes in cellular morphology

associated with projection of highly ramified processes, which progressively wrap and insulate axons at intervals delimited by internodes.

Myelination depends on the temporal coordination of numerous processes in various cell types, including OPCs, mature oligodendrocytes, and neurons (Mitew *et al.* 2013). Over the years, a growing list of factors have been identified that influence myelination by regulating such processes, including proteins involved in the control of cell proliferation and gene transcription, as well as others with roles on both extracellular and intracellular signaling (Fekete and Nishiyama 2022). Among the latter are various protein kinases, including mitogen-activated protein kinases (MAPKs) in the c-Jun amino-terminal kinase (JNK) pathway module (Gaesser and Fyffe-Maricich 2016). Mammalian genomes feature three different genes encoding JNK kinases: JNK1, JNK2, and JNK3, which together give rise to ten different JNK isoforms (Davis 2000). While JNK1 and JNK2 are ubiquitously expressed in all tissues, JNK3 is primarily expressed in the brain (Bogoyevitch 2006). All JNK isoforms share major structural features and some common substrates (Coffey 2014). However, genetic and experimental evidence demonstrated isoform-specific distributions and roles of JNKs on the proliferation, development, maintenance, and death of different cell types (Bogoyevitch 2006; Yamasaki *et al.* 2012). Potentially relevant to the myelination process, all mammalian JNK isoforms are expressed in cells of the oligodendroglial lineage (Lorenzati *et al.* 2021; Li *et al.* 2007; Pirianov *et al.* 2006; Jurewicz *et al.* 2005), and ubiquitous genetic ablation of JNK1 in mice was found to enhance proliferation and branching complexity of OPCs in a cell-autonomous manner (Lorenzati *et al.* 2021). Despite this knowledge, whether other JNK isoforms impact these and other aspects of the myelination process *in vivo* has not been addressed before.

Guided by results from an unbiased proteomics analysis and taking advantage of mice featuring germline deletion of the JNK3 isoform ($JNK3^{-/-}$ mice), we report a deleterious impact of ubiquitous JNK3 deletion on the myelination of callosal axons. Ultrastructural analyses of the CC further revealed increased numbers of small caliber axons and thinner myelin sheets in $JNK3^{-/-}$

mice, compared to wild type mice (WT). Consistent with these findings, biochemical studies showed a marked reduction in the phosphorylation of neurofilaments (NFs), major cytoskeletal components involved in the regulation of axonal caliber. Collectively, findings from *in vivo* studies here establish a deleterious impact of ubiquitous JNK3 deletion on myelination, paving the path for studies aimed to reveal cell-type specific contributions of this kinase to the various events involved in this cellular process.

MATERIALS AND METHODS

Animals. All animal procedures were performed in accordance with IAUAC and institutional animal protocol guidelines at the University of Illinois (Animal Protocol 23-076). Mice were housed in individually ventilated cages with littermates of mixed genotype (3-5 mice per cage). Mice were maintained on a reverse 12h light/dark cycle with free access to water and food throughout the study. Wild type (WT; JAX 000664) and homozygous JNK3 knockout mice ($JNK3^{-/-}$ mice; C6.129S1-Mapk10tm1Flv/J; JAX 004322) were obtained from Jackson Laboratories and crossed for over ten generations to obtain $JNK3^{-/-}$ mice with a pure C57BL/6 background. A description of the breeding strategy and numbers of mice used in this study is provided in **Suppl. Fig. 1**.

Preparation of callosal tissue samples. At age P45, mice were euthanized by CO_2 inhalation, After cessation of respiration, mice underwent quick cervical dislocation. Brains were dissected and placed on an ice-cold mouse brain matrix grid (Zivic Instruments, catalog number 5324). For each mouse, two 1 mm sagittal slices (immediately adjacent to the midline) were obtained using razor blades and placed on the surface of an ice-cold glass. Tissue corresponding to the anterior and medial section of CC on both slices was manually dissected, pooled, snap-frozen in liquid nitrogen and stored at -80C until processing.

Proteomic sample preparation and mass-spectrometry. Frozen callosal tissue punches generated at the laboratory of Dr Morfini (University of Illinois, Chicago) were shipped on dry ice to Evotec for proteomic analyses (Munich, Germany). Each sample was lysed in Urea buffer: 8 M

Urea, 75 mM NaCl, 50 mM Tris, pH 8.2, 10 mM Sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, Roche Protease Inhibitor tablet, 2 mM Na_3VO_4, 10 mM beta-Glycerophosphate, phosphatase inhibitor cocktail 2 (PIC2, Sigma) and phosphatase inhibitor cocktail 3 (PIC3, Sigma), disrupted by sonication and cleared by centrifugation. The minimum acceptable amount of protein obtained for mass spectrometry-based analysis was 120 µg. Lysates were reduced, alkylated and proteolytically digested using the proteases LysC and Trypsin. The resulting peptides were desalted and differentially isotope-labeled with TMT (126, 127N, 127C, 128N, 128C, 129N, 129C, 130N, 130C, 131). For all samples, TMT labeling efficiencies were determined and found to be higher than 95% throughout the study. Subsequently, equal amounts of TMT-labeled peptides were combined into a TMT 10-plex experiment. Samples were lyophilized, desalted, and the complex mixture of tryptic peptides thereafter fractionated into a total of 10 fractions by high pH-reversed phase chromatography using an X-Bridge column on an ÄKTA FPLC system. Upon desalting, an aliquot was taken from each fraction and subsequently employed for total proteome analysis. Samples were then analyzed by liquid-chromatography tandem mass-spectrometry on a Thermo Q Exactive HF mass spectrometer equipped with an Easy nLC-1000 UPLC system (Thermo Fisher Scientific). Samples were loaded with an autosampler onto a 40 cm fused silica emitter (New Objective) packed in-house with reversed phase material (Reprusil-Pur C_{18}-AQ, 1.9 µm, Dr. Maisch GmbH) at a maximum pressure of 950 bar. The bound peptides were eluted over 120 min run time and sprayed directly into the mass spectrometer using a nanoelectrospray ion source (ProxeonBiosystems).

Proteomics data processing. The 300 RAW files were collectively processed using the MaxQuant (Tyanova *et al.* 2016) software (version 1.6.0.15) and searched against a reference murine proteome database (swissprot_mouse_2017_09) with parameters set to ensure an FDR of < 1%. All peptides were required to have a minimum peptide length of five amino acids and a maximum of two missed cleavages. Carbamidomethylation of cysteine was set as a fixed

modification and oxidation of methionine, protein N-acetylation were allowed as variable modifications. The match between runs option was enabled. During MaxQuant processing, results from each of the fractions were collapsed resulting in experiments for each genotype and tissue that were used for the further analysis.

MaxQuant “proteinGroups' tables were used to analyze protein quantification. For all statistical analyses, MaxQuant intensities were log10-transformed and features flagged as reverse or contaminant hits were excluded. To adjust for loading effects, a scaling factor was subtracted from each sample. The scaling factor is the difference of the median intensity for each sample and the overall median intensity with missing values excluded from median computation. For the principal component analysis (PCA) only proteins that had no missing values were considered. A “virtual pool” was then created by calculating the median intensity of each protein for a TMT 10-plex experiment. For normalization, the virtual pool was subtracted from the log10-transformed scaled intensities for each sample of the corresponding TMT 10-plex. These ratios over the virtual pool were then median centered. No outlier detection was performed, and the following analyses were conducted on these data.

Differential protein abundance analysis. The normalized reporter intensities were corrected for batch effects using ComBat in the sva R package (v3.38). For principal component analysis (PCA), batch-corrected reporter intensities were transformed using the variance stabilizing transform function in the vsn R package (v3.68.0). PCA was then performed on the vsn-transformed intensities using prcomp in R. Differential protein abundance analysis in the corpus callosum of WT versus JNK3$^{-/-}$mice (n=9 per group, no sample size calculation was done (Levin 2011) was performed using the limma R package (v3.44.1) with eBayes. The statistical significance threshold was set at BH-adjusted p-value≤0.10. Oligodendrocyte-specific proteins were downloaded from Sharma et al (Sharma *et al.* 2015) and compared with the list of differentially abundant proteins identified at stringent thresholds of BH-adjusted p-value≤0.05.

Transmission electron microscopy. Transmission electron microscopy (TEM) imaging and analysis was performed by the laboratory of Dr Pouladi. Briefly, animals were perfused at 45 days of age with ice-cold PBS followed by ice-cold 2.5% PFA and 2.5% glutaraldehyde (GlutAH) in PBS. Brains were removed and left in 2.5% PFA and 2.5% GlutAH for 24 h, washed in PBS and then transferred to a 5% sucrose solution containing 0.08% sodium azide in PBS at 4 °C until use. A coronal slice of 1 mm was made at the intersection between the CC and fornix using a mouse brain stainless steel matrix (Roboz Surgical Instrument Company). According to the mouse brain atlas (PMID: Paxinos.2004), the anterior region of the CC (from bregma 1.10 to 0.50 mm) was microdissected before post-fixation in 1% osmium tetroxide for 1 h at room temperature. After washing twice in deionized water, samples were dehydrated, infiltrated with acetone and resin, embedded and polymerized at 60 °C for 24 h. Ultra-thin slices (90 nm) were sectioned before imaging on a transmission electron microscope. Samples were viewed using a Tecnai G2 Spirit Twin/ Biotwin model (FEI, USA) and 12-16 images were taken from each animal. Images were taken by an investigator blinded to genotype and treatment.

G-ratio analysis. A total of 12 images per animal were analyzed using ImageJ software (version 2.0.0). For g-ratio analysis, a frame (region of interest) was randomly positioned on each electron microscopy image to eliminate selection bias. This systematic random sampling approach ensures that axons are selected based on objective spatial criteria, rather than investigator preference, providing representative measurements across the entire corpus callosum area analyzed. Within the selected region of interest, the axonal diameter (ID) and outer diameter (OD) of regular shaped and myelinated axons was measured and g-ratios calculated. Approximately a total of 300 axons per animal were measured for the anterior region of the corpus callosum analysis. G-ratio analysis was performed by an investigator blinded to genotype and treatment, as before (Teo *et al.* 2016).

Immunohistochemistry. Preparation of brains for immunohistochemical studies was done at UIC. At age P45, mice were anesthetized with Ketamine (150 mg/kg) and Xylazine (10 mg/kg),

and transcardially perfused first with PBS (10 mls), followed by PBS containing 4% paraformaldehyde (PFA, 20 mls). Brains were dissected and pos-fixed with 4% PFA for 16hrs, then rinsed in TBS, cryoprotected through washes in sucrose solutions of increasing concentration (5%, 10%, 20% and 30% sucrose in TBS), and stored at 4°C. Fixed brains were shipped to UBC, where they were processed as in our published studies (Bardile *et al.* 2019). Briefly, brains were embedded in OCT compound, fast-frozen in dry ice plus isopentane and stored at -80°C until sectioning. Coronal brain sections (40 µm) were prepared using a Leica Cryostat 3050 and stored in TBS containing 0.02% sodium azide. Free-floating sections were permeabilized in 0.5% Triton-X (w/v) in TBS, blocked in Blocking Buffer (1% BSA, 0.1% Triton-X, 1% Gelatin, 0.3M and 0.5% Goat Serum in TBS), and then incubated with primary antibodies: rabbit anti-NG2 (Sigma-Aldrich catalog number AB5320, 1:500), rabbit anti-Olig2 (Chemicon catalog number AB9610, 1:750) or rabbit anti-GSTpi (MBLI catalog number 311, 1:1000). Primary antibodies were diluted in PBS containing 5% normal goat serum (Sigma) and 0.2% Triton X-100 in PBS. After overnight incubation at 4 °C, sections were washed in PBS and incubated with biotinylated anti-rabbit antibody (1:200; Vectastain ABC HRP Kit, PK-4006; Vector Laboratories, Burlingame, CA, USA) for 1.5 h at room temperature in PBS containing 1% goat serum and 0.2% Triton X-100. After three washes in PBS, sections were incubated in Vectastain Elite ABC reagent (Vector Labs Inc., Burlingame, CA, USA) for 2 h at room temperature. Antibody immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB) (ImmPACT DAB Peroxidase Substrate, SK-4105; Vector Laboratories, Burlingame, CA, USA). Sections were mounted on slides and cover slipped with DPX mounting media (Sigma, 44581). Representative images of callosal tissue (WT) stained with anti-GSTpi and anti-NG2 antibodies are shown in **Supplementary Figure 2**.

Stereology-based quantitation of oligodendroglial cells. Densities of NG2-, Olig2-, and GSTpi-positive cells in the corpus callosum were determined by examining both left and right hemispheres of four coronal sections/mouse, as before (Bardile *et al.* 2019; Radulescu *et al.* 2019; Radulescu *et al.* 2024). The corpus callosum was defined as the white matter tract

extending between the lateral ventricles, bounded anteriorly by the genu, posteriorly by the splenium, dorsally by the cingulum, and ventrally by the septum pellucidum. The coronal navigation of the mouse brain atlas (Allen Mouse Brain Reference Atlas; http://www.brain-map.org) was used as a reference to draw contours for the corpus callosum region of interest. Sections were selected at regular intervals spanning from approximately Bregma -1.82 to -2.70 mm. Analysis was done using the Stereo Investigator Software (MBF Bioscience, Williston, VT, USA) with optical fractionator (cells counts) or Cavalieri probe (area) connected to an AxioImager M2 microscope (Carl Zeiss AG, Oberkochen, Germany) and AxioCam MRc Digital CCD camera (Carl Zeiss AG, Oberkochen, Germany). The following parameters were used: 50 × 50 µm counting frame size and 250 × 150 µm grid size for NG2, Olig2 and GSTpi. For all stereological measurements, a 10-µm dissector height and 5µm guard were used. Gunderson coefficients of error (CE) were calculated for all stereological counts, with mean CE M0 values < 0.10 for all measurements. For all analyses, the investigator was blinded to genotypes.

Immunoblotting-based analysis of neurofilament phosphorylation. Callosal tissues were dissected from mice (age P45), as done for proteomics analyses (**Suppl. Figure 1**). Samples were homogenized in Lysis Buffer [LB: Hepes 20mM, 1%SDS, 2mM sodium orthovananadate, and 10mM sodium fluoride (pH7.4)]. For each mg of wet tissue, 100µl of LB were used. Following homogenization, samples were heated to 65C for 10 minutes, and DNA broken by brief sonication. Homogenates were clarified by centrifugation (15,000gmax for 5 minutes). Supernatants were collected, transferred to fresh tubes, and spun again. Protein concentrations in supernatants were determined using a BCA assay (PierceTM BCA Protein Assay kit, catalog number 23225). Lysates were normalized to the same protein concentration (2µg/µl) using LB, and a 6X SDS sample buffer stock added for a final 2X concentration. Samples were separated using 4-12% NuPAGE Novex 4-12% Bis-Tris Gels (Invitrogen). Proteins were transferred to PVDF membrane (Merk-Millipore cat IPFL00010) using Towbin buffer plus 10% methanol. Membranes were rinsed and blocked in TBS containing 1% w/v nonfat dry milk (Nestle) for 1 hour. Primary

antibodies were diluted in TBS containing 1% BSA and incubated overnight at 4C. Primary antibodies included: rabbit anti-JNK3 (clone 55A8, Cell Signaling catalog number 2305), mouse anti-synaptophysin (clone SVP38, Santa Cruz catalog number 12737), rabbit anti-NFL (clone C28E10, Cell Signaling, catalog number 2837); and phosphorylation-dependent mouse antibodies recognizing NFH (clone SMI31, Covance catalog number SMI-31R; clone SMI32, Covance catalog number SMI-32R; and clone RT-97, DSHB catalog number RT97), Secondary goat anti-mouse and goat anti-rabbit antibodies (Li-COR) were diluted in TBS containing 1% w/v dry milk and incubated 1h at room temperature. All blocking and antibody incubation solutions contained 2mM Sodium Orthovanadate and 10mM Sodium Fluoride to block contaminating phosphatases in milk. After washing, membranes were imaged using a Li-COR Odyssey infrared imaging system. Li-COR ImageStudioLite 5.2 Software was used to quantify immunoreactivity of bands.

Statistical analysis. Unless otherwise indicated, statistical analyses were conducted using Prism 6 (GraphPad Software), and data are presented as mean ± SEM. Sample size calculations were not performed a priori; sample sizes were determined based on prior studies of a similar nature (Hill et al., Nat Neurosci. 2018;21(5):683-695; VonDran et al., Glia 2010;58(7):848-856; Chittajallu et al., J Physiol. 2004;561(Pt 1):109-122; Desai et al., Am J Pathol. 2010;177(3):1422-1435). Data distributions were assessed for normality using the Shapiro-Wilk test prior to parametric analyses; unless otherwise noted, all datasets analyzed using Student's t-tests met the assumption of normality (Shapiro-Wilk test, $p > 0.05$). Statistical significance between two experimental groups was evaluated using Student's t-test, with $p < 0.05$ considered significant. No formal tests for outliers were performed for datasets analyzed using Student's t-tests, and no data points were excluded. Differential protein abundance analysis was performed using the limma R package (v3.44.1) with empirical Bayes moderation, and statistical significance was defined as a Benjamini-Hochberg-adjusted p-value ≤ 0.10.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno *et al.* 2016) partner repository with the dataset identifier PXD054532 (Username: reviewer_pxd054532@ebi.ac.uk; Password: IWVXR60xfMrJ). All other data that support the findings of this study are available from the corresponding authors upon reasonable request.

RESULTS

Quantitative proteomics analysis reveals increased expression of myelin-related proteins in the corpus callosum of JNK3$^{-/-}$ mice. Proteomic analysis of the corpus callosum (CC), the largest white matter structure in the brain, was concurrently done for wild type (WT) and JNK3-/- mice (*n*=9 and 10 samples/genotype, respectively, see **Suppl. Figure 1**). A total of 7,358 proteins were identified, where a principal components analysis (PCA) on the protein abundances showed a separation between control and JNK3$^{-/-}$ samples (**Fig. 1A**). We performed a differential protein abundance analysis and identified 538 differentially abundant proteins (FDR < 10%), with 29 presenting with an absolute $\log_2$FC > 1 (**Fig.** 1B and **Suppl. Table 1**). Among those proteins, several major myelin components were identified including myelin proteolipid protein (Plp1), claudin-11 (Cldn11), and myelin-oligodendrocyte glycoprotein (Mog), which were upregulated in JNK3$^{-/-}$ mice, compared to WT mice. We then compared our robustly different proteins (FDR<5%) with a previously published list of oligodendrocyte specific proteins (Sharma *et al.* 2015) and identified 50 that overlapped (**Fig. 1C**). Collectively, data for proteomics analysis strongly suggested that genetic JNK3 deletion alters steady-state levels of myelin-related proteins in the CC *in vivo*.

Increased density of oligodendrocyte precursor cells, but not mature oligodendrocytes, in corpus callosum of JNK3$^{-/-}$ mice. Prompted by findings from the unbiased proteomics analysis

above, we examined whether altered expression of myelin-related proteins in JNK3$^{-/-}$ mice was associated with gross morphological changes of the CC at age P45 (**Figures 2A-C**). Forebrain weight was comparable between WT and JNK3$^{-/-}$ mice (**Fig. 2A**). Similarly, both total area (**Fig. 2B**) and volume (**Fig. 2C**) of the CC was similar between WT and JNK3$^{-/-}$ mice, ruling out potential atrophy or enlargement of this major white matter brain structure.

A prior study reported increased proliferation of OPCs in CC of mice lacking JNK1 (Lorenzati *et al.* 2021). Based on this precedent, we investigated whether genetic JNK3 deletion altered the density of specific oligodendroglial cell populations in the CC *in vivo* (**Figures 2D-F**). Towards this, we performed immunohistochemical studies using antibodies recognizing Olig2, NG2, and GSTpi to identify such populations (Suppl. Fig. 2). The oligodendrocyte transcription factor, Olig2, can be used to identify pan oligodendroglia cells, since it is expressed throughout developmental stages (Lu *et al.* 2002), and it is downregulated only at the late stage of oligodendrocytes maturation (Ligon *et al.* 2006). The proteoglycan NG2 is expressed in OPCs, but not in OPCs that mature towards the oligodendroglia lineage (Nishiyama *et al.* 2015). Differentiation into mature oligodendrocytes is associated with expression of glutathione S-transferase of class pi (GST-pi; (Cammer *et al.* 1989). Stereology-based quantitation methods revealed a significant increase in the density of Olig2- and NG2- positive cells in the CC of JNK3$^{-/-}$ mice, compared to WT mice (**Figs. 2D** and **E**, respectively). In contrast, the density of GSTpi-positive cells was similar among these experimental groups (**Fig. 2F**). Collectively, these results suggest that the alterations in myelin-related proteins observed in JNK3$^{-/-}$ mice are not associated with changes in CC volume or the density of mature oligodendrocytes in this structure. Instead, our results extended the repertoire of JNK isoforms that, when deleted, increase the density of OPCs *in vivo*.

Decreased myelin thickness and a shift in caliber distribution toward smaller axons in JNK3$^{-/-}$ mice, To directly investigate potential effects of JNK3 deletion on myelin formation, we

processed callosal tissues from ~~adult~~ WT and JNK3$^{-/-}$ mice for electron microscopic analysis. Axonal characteristics and myelin ultrastructure were analyzed as in our prior studies (Teo *et al.* 2016; Bardile *et al.* 2019; Radulescu *et al.* 2024) (**Figure 3A**). Quantitative analyses revealed no statistically significant differences in the number of myelinated (**Fig. 3B**) and unmyelinated axons (**Fig. 3C**) between WT and JNK3$^{-/-}$ mice

Next, we quantified myelin thickness by obtaining g-ratios, a measure of myelin sheath thickness representing the ratio between axoplasm and myelinated fiber diameter (caliber). Quantitation of g-ratios in regularly-shaped myelinated axons revealed no significant differences between WT and JNK3$^{-/-}$ mice (**Fig. 3D**). Because axonal caliber can influence myelination by regulating both myelin sheath formation and growth (Bechler *et al.* 2015; Lee *et al.* 2012; Mayoral *et al.* 2018), we plotted the relative frequency of axons in function of their diameter. This analysis revealed a different caliber distribution of myelinated callosal axons in JNK3$^{-/-}$ mice, compared to WT mice. Specifically, JNK3$^{-/-}$ mice showed increased numbers of small caliber myelinated axons (< 800μm diameter), a marked reduction in the number of large caliber myelinated axons (> 800μm diameter) and decreased average diameter of total myelinated axons (**Fig. 3E-F**). To examine variability of myelin sheath thickness as a function of axon caliber, we binned measured g-ratio values for three axonal caliber ranges: small (< 800 nm), mid-range (≥800, < 1600 nm diameter), and large (≥ 1600 nm diameter). This analysis showed increased mean of all g-ratios of callosal axons from JNK3$^{-/-}$ mice, compared to WT mice, indicating that myelin sheaths of axons with small and mid-range caliber are thinner in JNK3$^{-/-}$ mice (**Fig. 3G-H**). Cumulative frequency plot of all g-ratios for two axon size groups confirmed a robust shift towards larger g-ratio values in JNK3$^{-/-}$ mice. Compared to WT mice, a marked increase in the percentage of small caliber myelinated axons (14% *versus* 81%) (**Fig. 3I**) and a concomitant decrease in the number of large diameter myelinated axons (19% *versus* 1%) was observed in JNK3$^{-/-}$ mice. Collectively, results from ultrastructural analysis revealed a higher proportion of small caliber callosal axons with

thinner myelin sheaths in JNK3$^{-/-}$ mice, indicating that genetic JNK3 deletion affects myelinogenesis and/or myelin maintenance.

Reduced phosphorylation of neurofilaments in corpus callosum of JNK3$^{-/-}$ mice. Selectively expressed in mature neurons, neurofilaments (NFs) represent the most abundant cytoskeletal component providing the structural support of axons (Yuan *et al.* 2017). NFs are filamentous polymers mainly composed of heavy (NFH), medium (NFM), and light chain subunits (NFL) (Yuan *et al.* 2012). Incorporation of NFH and NFM subunits to the NF backbone depends on the NFL subunits, which act as seeds (Yuan *et al.* 2017). Phosphorylation of NFH and NFM subunits at numerous KSPXK and KSPXXXK motifs in their C-terminal tail domains causes projection of these domains away from the NF backbone, increasing radial growth of NF polymers (Rudrabhatla and Pant 2010). Relevant to data in **Fig. 3E-F**, compelling evidence has linked the phosphorylation state of NFH and NFM subunits to the myelination state and caliber of axons (Sánchez *et al.* 2000). Further, JNKs have been listed as a subset of kinases that can directly phosphorylate KSPXK and KSPXXXK motifs in NFHs (Veeranna *et al.* 2011; Holmgren *et al.* 2012). Collectively, these precedents prompted us to evaluate potential alterations in NF phosphorylation in callosal tissue of JNK3$^{-/-}$ mice.

CC lysates were prepared from WT and JNK3$^{-/-}$ mice and processed for immunoblotting using antibodies that recognize phosphorylation-dependent epitopes on NFH subunits (**Fig. 4A**). Both RT-97 (Veeranna *et al.* 2008) and SMI-31 (Veeranna *et al.* 1998) are mouse monoclonal antibodies that specifically recognize subsets of phosphorylated residues KSPXK and/or KSPXXXK motifs of NFH subunits. Significantly, immunoreactivity of these antibodies was markedly reduced in CC lysates prepared from JNK3$^{-/-}$ mice, compared to those prepared from WT mice. In contrast, an antibody that recognizes a different, preferentially dephosphorylated epitope in NFHs (SMI-32 antibody (Campbell and Morrison 1989)) showed similar levels of immunoreactivity among these samples. Monoclonal antibodies recognizing synaptophysin and

NFL subunits confirmed similar levels of neuronal protein loading. These findings were confirmed by LICOR-based quantitative analysis of RT-97/NFL, SMI-31/NFL, and SMI-32/NFL immunoreactivity ratios (**Fig. 4B**).

DISCUSSION

Highly expressed in neurons and oligodendroglia, JNK3 remains the least studied mammalian JNK kinase isoform. Prior studies revealed a role for JNKs in the survival and function of oligodendroglia, and a contribution of the JNK1 isoform to the proliferation and differentiation of OPCs (Lorenzati *et al.* 2021), but potential contributions of the JNK3 isoform to myelogenesis have not been addressed before. Supporting such contribution, results from an unbiased proteomic analysis here revealed upregulation of myelin proteins in JNK3$^{-/-}$ mice, compared to WT mice. Building on these findings, we found that ubiquitous genetic-based ablation of JNK3 results in thinner myelin sheaths of callosal axons, increasing also the proportion of small-caliber callosal axons. Intriguingly, this phenotype was accompanied by increased density of OPCs, but not mature oligodendroglia. The apparent paradox of increased myelin protein expression without corresponding increases in mature oligodendrocyte density or myelin thickness suggests several possible mechanisms. First, the upregulation in levels of myelin proteins may represent a compensatory response to the deficits in myelination we observed, where existing oligodendrocytes increase protein production to offset the functional impairment (Ishii *et al.* 2012). Alternatively, JNK3 deletion may impair the final stages of oligodendrocyte maturation or myelin sheath formation, leading to increased protein synthesis without proportional myelin deposition. The observation that JNK3-/- mice exhibit no change in the density of mature GSTpi+ oligodendrocytes, yet exhibit thinner myelin sheaths on smaller axons, suggests that JNK3 deletion may impair the functional capacity of oligodendrocytes, rather than their differentiation *per se* (Fyffe-Maricich *et al.* 2013). This could indicate that while oligodendrocytes successfully differentiate and express mature markers like GSTpi, their ability to generate appropriate myelin

sheath thickness is compromised. This functional deficit may be particularly pronounced for smaller caliber axons, which may require more precise oligodendrocyte-axon signaling to achieve optimal myelination (Câmara *et al.* 2009). A limitation of our study is the lack of proliferation marker analysis to determine whether increased OPC density results from enhanced proliferation at P45 or from developmental alterations earlier in life. Moreover, because our analyses were limited to a single age (P45), it remains possible that the observed differences may reflect a developmental delay or transient phenotype that could resolve in older mice. Thus, the possibility that JNK3 deletion leads to a delay, rather than a persistent defect, in maturation cannot be excluded with the current dataset. Future studies incorporating Ki67 immunohistochemistry co-localized with NG2 and Olig2 markers and additional time-points (>3 months of age) would clarify this important mechanistic question and help distinguish between ongoing proliferative effects *versus* developmental programming changes resulting from germline JNK3 deletion. In sum, although our observations suggest a potential role of JNK3 in the differentiation of OPCs, ubiquitous deletion of this kinase in JNK3$^{-/-}$ mice could not rule out a contribution of additional cell types to altered myelination.

Throughout normal mouse brain development, the diameter of axonal fibers gradually expands in an oligodendroglia- and myelination-dependent manner (Nowier *et al.* 2023). This increase in axonal caliber is associated with heightened phosphorylation of NFH and NFM subunits of NFs (Nowier *et al.* 2023), the most heavily phosphorylated proteins in neurons (Pant and Veeranna 1995; Holmgren *et al.* 2012). Interestingly, our data revealed decreased phosphorylation of NFs at sites recognized by the SMI31 and RT97 phosphoantibodies (Sánchez *et al.* 2000). This finding is consistent with prior studies revealing JNK3 as one of several proline-dependent protein kinases capable of phosphorylating NFH subunits at residues in the KSPXK and/or KSPXXXK motifs, including those recognized by the RT-97 antibody (Veeranna *et al.* 2008; Nowier *et al.* 2023). An impact of JNK3 deletion on NF phosphorylation could therefore explain the higher number of small caliber axons found in the CC of JNK3$^{-/-}$ mice (Nakano *et al.* 2020).

Further, increased ~~number~~ density of OPCs in these mice could reflect an oligodendroglial response to axonal alterations associated with JNK3 deletion.

While NFs are crucial for maintaining axonal structure and the control of diameter, myelination, in turn, can influence axonal caliber (Hsieh *et al.* 1994; Martini 2001). The initial evidence demonstrating the influence of oligodendrocytes on axonal diameter came from a study where the development of OPCs was prevented by X-irradiation. The absence of oligodendrocytes led to a significant reduction in the mean axonal caliber in the optic nerve (Colello *et al.* 1994). Further studies have shown that the mere ensheathments of axons by oligodendroglial processes is sufficient to stimulate axonal caliber expansion (Sánchez *et al.* 1996). This process has been shown to involve accumulation, reorganization, and phosphorylation of neurofilaments (Sánchez *et al.* 1996). In the cuprizone mouse model of demyelination, Manson and colleagues observed a notable decrease in the mean axonal caliber during the acute phase of demyelination, while, upon remyelination, the axonal caliber returned to normal (Mason *et al.* 2001). In cases where mice were kept on a cuprizone diet for an extended period, preventing remyelination, the axonal diameter further decreased, reaching up to 60% of the normal size (Mason *et al.* 2001). Collectively, these studies emphasized the intricate interplay between axonal caliber, neurofilament phosphorylation, and the myelination process. Within this context, findings from our work here indicates that JNK3 is necessary for proper myelination of ~~adult~~ callosal axons *in vivo*, highlighting a potential role of this kinase in the intricate system that regulates oligodendroglia function and myelination.

In sum, the analyses of $JNK3^{-/-}$ mice in this study strongly supports a role of JNK3 as a regulator of callosal axon myelination and caliber. Future studies involving selective ablation of JNK3 in these cell types will elucidate cell-autonomous contributions of this kinase on the myelination process.

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COMPETING INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. An unbiased proteomics analysis reveals increased expression of numerous myelin-related proteins in the corpus callosum of JNK3 knockout mice, compared to wild type mice. A) Principal component analysis (PCA) for the normalized TMT protein abundances in wild type (WT, blue, n=10) and JNK3 knockout ($JNK3^{-/-}$, orange, n=9) mice. **B**) Volcano plot of the differentially abundant proteins between WT and $JNK3^{-/-}$. Significantly upregulated (red) and downregulated (blue) proteins are indicated (empirical Bayes moderated t-statistics, FDR < 10%, absolute log fold change > 1). **C**) Heatmap of proteins with stringent, significantly different abundances between WT and $JNK3^{-/-}$ (FDR < 5%), previously identified as enriched in oligodendroglial cells (Sharma *et al.* 2015).

Figure 2. Increased number of oligodendrocyte precursor cells (OPCs), but not mature oligodendrocytes, in corpus callosum of JNK3 knockout mice. A-C) No differences in forebrain weight (**A**; $t(7)=1.591$, $p=0.1556$), corpus callosum area (**B**; $t(7)=0.2895$, $p=0.7806$) or corpus callosum volume (**C**; $t(7)=0.3340$, $p=0.7482$) were observed between wild-type (WT) and

JNK3 knockout (JNK3$^{-/-}$) mice. **D-F**) Density (cells/mm²) of NG2-, Olig2-, and GST-pi expressing cells in corpus callosum. Representative images of callosal tissue (WT) stained with anti-GSTpi and anti-NG2 antibodies are shown in **Supplementary Figure 2**. A significant increase in the number of NG2- (**D**; t(7)=2.408, p=0.0469) and Olig2-positive cells (**E**; t(7)=3.081, p=0.0178) was observed in the corpus callosum of JNK3$^{-/-}$ mice, compared to WT mice. In contrast, no difference in the number of GSTpi-positive cells (**F**; t(7)=0.2602, p=0.8022) was observed among these genotypes. n= 4 WT and 5 JNK3$^{-/-}$ mice. Data represent means ± SEM; * P < 0.05; two-tailed unpaired Student's t-test.

Figure 3. Increased proportion of small caliber axons and thinner myelin sheets in corpus callosum of JNK3 knockout mice. A) Representative electron microscopy ages of myelinated axons in the corpus callosum of wild type (WT) and JNK3 knockout (JNK3$^{-/-}$) mice. No statistically significant differences were found in the numbers of myelinated (**B**; t(4)=1.318, p=0.2580) and unmyelinated axons (**C**; t(4)=0.8492, p=0.4436) between WT and JNK3$^{-/-}$ mice. **D**) G-ratios of regular shaped myelinated axons in WT mice and JNK3$^{-/-}$ mice. (**E, F**) Compared to WT mice, inner axonal diameters in JNK3$^{-/-}$ mice showed a different distribution, presenting more axons with a smaller caliber diameter (**E**) and overall decreased mean of inner axonal diameter (**F**; t(4)=14.15, p=0.0001). (**G, H**) Classification of all g-ratios in small (< 800 nm), and mid-range (800 ≤ diameter < 1600 nm) axonal diameters revealed higher g-ratios (thinner myelin sheaths) in JNK3$^{-/-}$ mice, compared to WT mice for both (**G**) small (t(4)=3.225; p=0.0321) and (**H**) mid-range diameter axons (t(4)=3.004; p=0.0398). Cumulative frequency plots represent all g-ratios for the two axon size groups. **I**) Pie chart representation of small, mid- and large size myelinated axons. ~300 axons were quantified per animal. Data show means ± SEM; *P < 0.05, ***P < 0.001; two-tailed Student's test; scale bar= 1 µm.

Figure 4. Reduced phosphorylation of neurofilament heavy chain subunits in JNK3 knockout mice. A) Immunoblotting analysis of corpus callosum lysates prepared from wild type (WT, n=7) and JNK3 knockout (JNK3$^{-/-}$, n=6) mice. Immunoblots were developed using antibodies RT-97 and SMI-31, which specifically recognize phosphorylated KSPXK and/or KSPXXXK motifs in neurofilament heavy chain subunits (NFH). An antibody that recognizes a different, dephosphorylated epitope in NFH was also used (SMI-32). Phosphorylation-independent antibodies recognizing neurofilament light chain subunits (NFL) and synaptophysin (synaptoph) confirmed similar levels of neuronal protein loading. An antibody recognizing JNK3 was used to confirm genotypes for each sample. **B**-**D**) Quantitation of immunoblots in Panel A. Data represent means ± SEM; **P < 0.01, ***P < 0.001; two-tailed unpaired Student's t-test. Quantitative analysis of RT-97/NFL [**B**; (t(11)=3.602; p=0.0042)], SMI-31/NFL [**C**; (t(11)=5.113; p=0.0003)] and SMI-32/NFL [**C**; (t(10)=1.238; p=0.2440) immunoreactivity ratios in callosal tissues of WT *versus* JNK3 mice. Collectively, these data indicate reduced phosphorylation of NFH subunits at KSPXK and/or KSPXXXK motifs in callosal axons from JNK3$^{-/-}$ mice, compared to WT mice.

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